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ESTROGEN STIMULATION OF THE OVIDUCT IN VITAMIN-DEFICIENT CHICKS[1]

IRENE T. KLINE[2] AND RALPH I. DORFMAN[2a]
with the technical assistance of Patricia McCreery

From the Departments of Biochemistry and Medicine, Western Reserve University School of Medicine and Lakeside Hospital, Cleveland, Ohio

PROLIFERATION of the chick oviduct in response to stimulation by the synthetic estrogen, stilbestrol, is dependent upon the dietary level of pteroylglutamic acid (PGA) but is independent of the level of pyridoxine, pantothenic acid, or riboflavin (Hertz, 1945). We have repeatedly confirmed the point that optimal growth of the chick oviduct in response to stilbestrol stimulation does not occur in the absence of adequate amounts of PGA (Kline and Dorfman, 1948). In addition, we have found that stimulation of the chick oviduct by the natural estrogen, estrone, is also impaired by PGA deficiency.

The studies reported in this paper deal primarily with an investigation of the relation of single vitamin deficiencies to the action of stilbestrol over a range of dose levels. The main effort has been to find a means of differentiating between the effect of a vitamin deficiency in itself and the effect of the inevitable concomitant inanition on the response of the chick oviduct to stilbestrol stimulation. Even though, of experimental necessity, the approach to the problem has been that of single vitamin deficiencies, it should be recognized that a specific vitamin deficiency cannot be developed without creating an imbalance of the mechanisms involving other vitamins. Elucidation of such imbalances remains for future research.

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[1] This investigation was supported in part by a grant from the Nutrition Foundation.

[2] Sigma Delta Epsilon Fellow, 1949–1950. Now at Yale University School of Medicine.

[2a] Present address: The Worcester Foundation for Experimental Biology, Shrewsbury, Massachusetts.

As a working hypothesis we have considered three ways in which the lack of a specific vitamin may influence the response of the chick oviduct to estrogen. First, a vitamin deficiency may be evidenced in a decreased ability of the oviduct to proliferate in response to hormonal stimulation, with the implication that the vitamin, or its biologically active form, may have become a limiting factor in the enzyme system through which the estrogen exerts its effect. Second, the rate of the inactivation of the estrogen may be decreased if the vitamin of which there is a deficiency is involved in the biochemical mechanism through which the inactivation is accomplished. In this event, a given dose of estrogen is able to produce a response greater than would occur in an animal on a complete diet in which the inactivating mechanism is operating normally. Third, in any case, whether or not the vitamin deficiency results in an increased or decreased response to estrogen, the lack of a particular vitamin results in a decreased food intake and a lower rate of general body growth, i.e., a state of inanition, which is shared by the oviduct. This decreased growth rate becomes more evident in the oviduct upon attempts at hormonal stimulation, as a nonspecific, reduced sensitivity or increased resistance to stimulation, which has already been found to be characteristic of the mammary gland of rats on limited food intake (Trentin and Turner, 1941). In order to evaluate various vitamins with reference to their mode of involvement in the action of estrogen, it has been necessary, therefore, to take into consideration the degree of inanition present in any deficiency state. It is conceivable, then, that a vitamin deficiency may operate in three ways to influence the response of the oviduct to estrogenic stimulation and that any observed response may or may not be the resultant of all three types of involvement operating simultaneously.

MATERIALS AND METHODS

White Leghorn or Sex-linked Cross pullets, obtained from Kerr Chickeries, Frenchtown, New Jersey, were used. They were received in the laboratory 24 to 48 hours after hatching and were thereafter maintained on a synthetic diet containing all known vitamins (Hertz, 1945) except in those cases in which a vitamin was omitted deliberately or in which a commercial starting mash (Conkey's YO) was used for comparison with the complete synthetic diet. The synthetic diet consisted of a purified basal ration and an aqueous supplement. The ingredients of the basal diet per 100 grams were: vitamin-free casein, 25.0 gm.; gelatin, 10.0 gm.; l-cystine, 0.3 gm.; choline chloride, 0.2 gm.; corn starch, 52.4 gm.; cellulose, 2.0 gm.; Crisco, 4.0 gm.; salts (Briggs *et al.*, 1943), 6.0 gm.; α-tocopherol, 0.025 gm.; Vit. A, 1,800 U.S.P. units; Vit. D, 255 units. The aqueous mixture given orally, daily in the amount of 0.06 ml. contained: calcium d-pantothenate, 75 μg.; riboflavin, 60 μg.; nicotinic acid, 125 μg.; i-inositol, 2.5 mg.; thiamine hydrochloride, 50 μg.; p-aminobenzoic acid, 0.75 mg.; pyridoxine hydrochloride, 50 μg.; 2-methyl-1,4-naphthoquinone, 0.5 μg.; biotin, 1.5 μg.; pteroylglutamic acid (PGA), 0.1 mg.; Cobione (Vitamin B_{12}), when included, 0.1 μg.

Vitamin deficiencies were produced by the simple expedient of omitting single vitamins from the synthetic diet except in the case of PGA.[3] In the early experiments even PGA deficiency was obtained in this manner, but it involved keeping the animals on the deficient diet for a long period or using animals with a less pronounced deficiency than was desired. For later experiments, a crude antagonist of PGA[5] (Franklin, *et al.*, 1947) was incorporated in the basal diet in order to produce a deficiency state in a shorter period of time. All animals on the synthetic diet received the same volume of the aqueous vitamin supplement whether or not the basal ration was supplied *ad libitum* or in limited amount, and whether or not one or more vitamins had been omitted from the mixture.

The estrogens[4] were dissolved in corn oil so that the total dose was contained in 0.5 cc. and were injected subcutaneously in five equal doses over a period of 5 days, the last injection having been made 24 hours prior to autopsy. At autopsy the oviducts were weighed immediately after dissection, on a Roller-Smith torsion balance. Any variations from this routine are indicated for individual experiments.

The "t" method of Fischer (1941) was employed to test significances of differences between means. In many tables the t value, a measure of the significancy, is included. For values of t below 2.09 for a total of 20 observations (total number of animals in the two groups compared), $P > 0.05$, which indicates no significant differences between the means. Values of t between 2.09 ($P = 0.05$) and 2.86 ($P = 0.01$) are considered to indicate a significant difference between means, while values of t greater than 2.86 ($P < 0.01$) indicate a highly significant difference between the means. In most of our comparisons a total of 20 or more animals were used. As the number of observations increases, the t value for the same level of significancy decreases. The P value indicates the probability of two samples being of the same population. For example, if the P-value is 0.05, there is only one chance in twenty that the two samples are from the same population.

RESULTS

PGA-Deficiency.—The data from six experiments with PGA-deficient chicks are presented in Table 1. In all instances, at all levels of estrogen dosage, animals deficient in PGA showed significantly lower oviduct stimulation than did the pullets on a diet adequate in its content of PGA. This is true not only for stilbestrol but also for the natural estrogen, estrone (Experiment 4).

Criteria by which it is possible to judge the presence of and, to some extent, the severity of a vitamin deficiency in chicks are a decreased rate of body growth or an actual loss of body weight. The mean body weights at autopsy of the groups on the complete and the PGA-deficient diets have been listed for comparison. In five of the six experiments, significantly

[3] PGA was supplied by the Lederle Laboratories Division, American Cyanamid Company.

[4] Stilbestrol was supplied by the Winthrop-Stearns, Inc., and the estrone, by Ciba Pharmaceutical Products, Inc.

[5] This substance represented the products of the condensation of 2,4,5-triamino-6-hydroxy-pyrimidine and p-amino-benzoyl-1(+)-glutamic acid with 2,3-dibromobutyraldehyde (Angier *et al.*, 1946). It was supplied to us by Dr. A. D. Welch of the Department of Pharmacology from material furnished by the Lederle Laboratories Division, American Cyanamid Company, through the courtesy of Dr. T. H. Jukes.

lower body weights in the PGA-deficient groups (except in the group receiving no stilbestrol in Experiment 9) indicate that the vitamin deficiency actually was obtained. In one experiment, Experiment 42, the animals on the complete diet were markedly stunted for reasons not completely understood.

That the body weights of the animals on the complete synthetic diet differ from experiment to experiment (not considering Experiment 42)

TABLE 1. OVIDUCT RESPONSE TO STILBESTROL IN PGA-DEFICIENT CHICKS

Exp. No. (Breed[a])	Days on diet	Estrogen total dose μg.	Complete synthetic diet			PGA-deficient diet			Mean body weight difference t[c]	Mean oviduct weight difference t
			No. of chicks	Mean body weight gm. ± S.E.[b]	Mean oviduct weight mg. ± S.E.	No. of chicks	Mean body weight gm. ± S.E.	Mean oviduct weight mg. ± S.E.		
		Estrone								
4 (W.L.)	23	0	10	128 ± 9	26 ± 1	7	82 ± 5	19 ± 1	4.5	0.4
		3000	10	128 ± 8	271 ± 36	7	90 ± 5	57 ± 9	3.1	5.8
		Stilbestrol								
10 (W.L.)	21	0	24	112 ± 3	19 ± 1	22	97 ± 2	18 ± 1	3.8	0.6
		1500	21	121 ± 4	785 ± 126	19	95 ± 2	121 ± 37	5.7	5.2
		Stilbestrol								
9 (W.L.)	14	0	20	84 ± 2	14 ± 1	21	81 ± 2	14 ± 1	1.3	0.1
		1500	21	93 ± 2	727 ± 42	22	76 ± 2	230 ± 22	3.2	6.2
		Stilbestrol								
42[d] (S.L.)	16	100	9	57 ± 4	53 ± 6	9	76 ± 5	37 ± 3	4.5	2.4
		200	9	52 ± 2	75 ± 8	13	67 ± 3	45 ± 4	4.5	6.8
		400	13	65 ± 4	174 ± 14	12	67 ± 3	61 ± 5	0.4	7.8
		1600	15	78 ± 4	497 ± 10	15	66 ± 3	120 ± 13	2.6	22.6
		2500	12	77 ± 2	608 ± 29	14	68 ± 3	141 ± 16	2.3	14.2
		Stilbestrol								
49[d] (S.L.)	16	0	10	119 ± 6	19 ± 2	15	72 ± 3	10 ± 0.1	7.6	4.5
		100	9	127 ± 7	78 ± 7	15	67 ± 2	36 ± 2	7.7	5.6
		200	10	125 ± 4	158 ± 10	14	80 ± 3	41 ± 4	8.3	11.4
		400	10	124 ± 5	315 ± 20	10	80 ± 4	77 ± 5	7.7	11.6
		1600	8	131 ± 3	678 ± 24	10	77 ± 3	95 ± 11	13.5	22.8
		3200	11	115 ± 5	775 ± 46	12	71 ± 4	105 ± 26	6.8	16.3
		Stilbestrol								
50[d] (W.L.)	16	0	11	95 ± 8	24 ± 5	12	59 ± 3	19 ± 3	4.1	0.9
		1750	12	110 ± 13	609 ± 46	10	59 ± 2	103 ± 18	3.8	10.2

[a] W. L. White Leghorn Breed; S.L.—Sex-linked Cross Breed.
[b] Standard Error of the Mean.
[c] Fischer's t value.
[d] Diet of PGA-deficient chicks contained 1% Lederle's crude PGA-antagonist.
In Experiments 4, 9, and 10, the hormone was given in 6 equal doses and autopsy was performed 24 hours after the last injection.
In Experiments 42 and 49, the hormone was given in 5 equal doses and autopsy was performed 24 hours after the last injection.
In Experiment 50, the hormone was given in 4 equal doses and autopsy was performed 48 hours after the last injection.

is explainable on the basis of differences in breed and in age at autopsy. In Experiments 4, 9, 10, and 50, a light weight breed of chicks, the White Leghorn breed, was used, while in Experiment 49 (and 42) a heavy breed, a Sex-linked Cross breed,[6] was used. The Sex-linked Cross pullets (Experiment 49) attain at 18 days of age a body weight approximately equal to that of the White Leghorns at 25 days of age (Experiment 4).

Comparison of the mean body weights of the PGA-deficient chicks to those of the controls on the complete synthetic diet gives some indication

[6] A cross between Barred Rocks and Rhode Island Reds.

of the severity of the deficiency of PGA developed in the different groups. In every case (except in Experiment 42 and the control group in Experiment 9) the difference in body weight was statistically significant since the value of P is less than 0.01. The t values are listed to furnish an index to the severity of the vitamin deficiency. When the crude antagonist was used (Experiments 49 and 50) actual loss of weight was observed in some of the animals before autopsy, although mean weights of the deficient groups showed a slight gain of about one gram per day (data not given). In Experiments 9 and 10 which were run concurrently and before the crude antagonist became available, the deficient animals continued to gain

TABLE 2. OVIDUCT RESPONSE TO STILBESTROL IN NICOTINIC ACID-DEFICIENT CHICKS

Exp. No. (Breed)	Days on diet	Stilbestrol total dose μg.	Complete synthetic diet			Nicotinic acid-deficient diet			Mean body weight difference t	Mean oviduct weight difference t
			No. of chicks	Mean body weight gm. ± S.E.	Mean oviduct weight mg. ± S.E.	No. of chicks	Mean body weight gm. ± S.E.	Mean oviduct weight mg. ± S.E.		
14 (W.L.)	36	1250	8	156 ± 7	841 ± 53	8	115 ± 5	581 ± 44	3.8	3.8
41 (S.L.)	14	100	9	101 ± 6	82 ± 4	12	82 ± 4	105 ± 9	2.7	2.2
		200	11	105 ± 4	154 ± 11	12	80 ± 4	187 ± 16	4.6	1.7
		400	10	97 ± 5	218 ± 18	12	70 ± 3	243 ± 10	5.0	1.2
		1600	9	102 ± 3	403 ± 35	14	76 ± 4	371 ± 22	5.4	1.0
50 (W.L.)	16	0	11	95 ± 8	24 ± 5	8	60 ± 4	15 ± 2	4.1	1.8
		1750	12	110 ± 13	609 ± 46	10	65 ± 4	437 ± 34	3.2	3.0

weight from the fourteenth to the twenty-first day at approximately three-fourths the rate of gain of the animals on the complete diet, or at 2.7 grams per day as compared to 4 grams per day. This occurred in the more slowly growing breed of chicks.

In general, the more severe the deficiency of PGA, the more limited is the proliferation of the oviduct in response to a specific dose of stilbestrol. This is evident in the t values for mean oviduct weight differences.

Experiment 42 is unique in that, although the animals on the complete diet were stunted so that the control and experimental groups had nearly the same body weights at autopsy, significantly lower oviduct weights were obtained in the PGA-deficient pullets.

Nicotinic Acid Deficiency.—In three experiments involving nicotinic acid deficiency (Table 2), the deficient animals all had significantly lower growth rates than did those on the complete synthetic diet. At the lower doses of stilbestrol, the deficient Sex-linked Cross pullets showed a tendency toward a greater oviduct response which was not apparent at a dose of 1,600 μg. Deficient White Leghorn pullets, however, were unable to respond as well to 1,250 and 1,750 μg. of stilbestrol as were the normal controls.

Riboflavin Deficiency.—Data from five experiments (Table 3) dealing with stilbestrol stimulation in riboflavin deficiency show that any given

TABLE 3. OVIDUCT RESPONSE TO STILBESTROL IN RIBOFLAVIN-DEFICIENT CHICKS

Exp. No. (Breed)	Days on diet	Stilbestrol total dose μg.	Complete synthetic diet			Riboflavin-deficient diet			Mean body weight difference	Mean oviduct weight difference	
			No. of chicks	Mean body weight mg. ± S.E.	Mean oviduct weight mg. ± S.E.	No. of chicks	Mean body weight gm. ± S.E.	Mean oviduct weight mg. ± S.E.	t	t	P
30 (S.L.)	16	1250	22	108 ± 3	457 ± 20	22	56 ± 2	299 ± 15	14.4	6.4	<0.01
32 (S.L.)	16	1250	12	108 ± 4	472 ± 34	12	62 ± 2	351 ± 20	10.2	3.02	<0.01
35 (S.L.)	15	1250	24	91 ± 10	426 ± 15	21	60 ± 2	314 ± 22	11.9	4.3	<0.01
37 (S.L.)	16	100	9	90 ± 6	71 ± 7	14	60 ± 2	47 ± 4	5.1	3.2	<0.01
		200	8	95 ± 3	120 ± 9	10	64 ± 3	107 ± 15	7.5	0.7	0.5
		400	9	95 ± 2	297 ± 21	10	55 ± 2	245 ± 22	4.2	1.7	0.1
		1600	9	97 ± 5	477 ± 25	11	55 ± 1	433 ± 37	7.6	1.0	0.3
50 (W.L.)	16	0	11	95 ± 8	24 ± 5	9	56 ± 2	17 ± 1	4.6	1.3	0.2
		1750	12	110 ± 13	609 ± 46	12	58 ± 1	450 ± 25	3.8	5.1	<0.01

dose of the hormone elicits a smaller response in deficient animals than it does in animals on a complete diet. In Experiment 37, there was no significant difference in oviduct weights produced in the two groups, except at the 100 μg. dose level even though body weights were significantly different. In Experiments 30, 32, and 35, using the Sex-linked Cross breed and in Experiment 50 with White Leghorns, significantly greater oviduct weights were produced by stilbestrol in the animals on the complete diet. The explanation for the discrepancy of results in Experiment 37 is not apparent. It is true that the animals on the complete diet did not attain the body weight which was expected for Sex-linked Cross breeds of this age. If failure to grow can be attributed to inanition, the oviduct weights should have been lower. Nevertheless, the animals on the complete diet in Experiments 30, 32, and 35 did not attain full body growth either. It seems more likely that the deficient animals of Experiment 37 gave a greater response to stilbestrol than did the deficient animals in Experiments 30, 32, and 35.

Thiamine Deficiency.—Two experiments were performed to study the

TABLE 4. OVIDUCT RESPONSE TO STILBESTROL IN THIAMINE-DEFICIENT CHICKS

Exp. No.	Stilbestrol total dose μg.	Complete synthetic diet			Thiamine-deficiency diet			Inanition diet		
		No. of chicks	Mean body weight gm. ± S.E.	Mean oviduct weight mg. ± S.E.	No. of chicks	Mean body weight gm. ± S.E.	Mean oviduct weight gm. ± S.E.	No. of chicks	Mean body weight gm. ± S.E.	Mean oviduct weight mg. ± S.E.
38[a]	60	11	105 ± 4	47 ± 2	6	44 ± 1	29 ± 4			
	120	11	101 ± 4	64 ± 3	8	41 ± 1	48 ± 5			
	240	10	102 ± 4	128 ± 14	6	45 ± 2	98 ± 9			
	960	10	104 ± 2	226 ± 26	6	45 ± 3	119 ± 15			
48[b]	100	8	141 ± 5	136 ± 26	7	71 ± 3	96 ± 10	11	69 ± 3	57 ± 5
	400	10	131 ± 6	350 ± 29	8	76 ± 1	311 ± 28	14	76 ± 2	260 ± 16
	1600	10	144 ± 6	666 ± 41	10	85 ± 3	727 ± 36	14	79 ± 3	574 ± 27

[a] Sex-linked Cross Breed; 14 days on diet; no thiamine was given to the thiamine-deficient groups.
[b] Sex-linked Cross Breed; 16 days on diet. Ten per cent of the amount of thiamine in the complete vitamin supplement was given to the thiamine-deficient groups for 11 days. During the last five days the allowance of thiamine was reduced to 5%.

influence of thiamine deficiency on the ability of the oviduct to respond to stilbestrol stimulation. In Experiment 38, Table 4, such a severe thiamine deficiency was produced that the animals had to be autopsied two days earlier than had been planned. The animals, therefore, received only three-fifths of the usual dose of hormone. The oviduct weights of the deficient animals were significantly lower than those of the animals on the complete diet, at all dose levels of stilbestrol.

In Experiment 48 (Table 4) the development of acute thiamine deficiency was prevented by administering ten per cent of the amount of thiamine present in the complete vitamin supplement until the day hormone injections were begun, when the allowance of thiamine was reduced to five per cent. Data which permit comparison to those obtained in the other

TABLE 5. COMPARISON OF OVIDUCT RESPONSES TO STILBESTROL IN CHICKS FED A COMMERCIAL MASH OR THE COMPLETE SYNTHETIC DIET*

Breed	Stilbestrol total dose μg.	Complete synthetic diet			Commercial mash			Mean body weight difference t	Mean oviduct weight difference	
		No. of chicks	Mean body weight gm. ± S.E.	Mean oviduct weight mg. ± S.E.	No. of chicks	Mean body weight gm. ± S.E.	Mean oviduct weight mg. ± S.E.		t	(P)
W.L.	0	12	100 ± 8	23 ± 2	7	114 ± 5	22 ± 1	1.5		
	3200	12	117 ± 7	844 ± 48	9	108 ± 3	908 ± 36	1.2	1.1	
S.L.	0	11	133 ± 7	29 2	10	138 ± 4	28 ± 3	0.6		
	3200	11	137 ± 6	1083 ± 51	12	132 ± 4	878 ± 55	0.7	2.8	(0.01

* Experiment 54; 18 days on diet.

experiments were thus obtained, the animals having survived well enough to receive the complete series of stilbestrol injections. Some of the animals had died of thiamine deficiency even before injections were begun, showing that ten per cent of the thiamine present in the complete vitamin supplement was a marginal amount for maintenance.

The data for Experiment 48 (Table 4) show that animals deficient in thiamine respond to a given dose of stilbestrol with oviduct proliferation not significantly different from that characteristic of animals receiving adequate amounts of thiamine, despite the fact that there is approximately a fifty per cent reduction in body weight in the deficient group. Comparison of the oviduct weights of the thiamine deficient animals to those of weight (inanition) controls, on the other hand, makes it evident that a thiamine deficiency permits a greater response to a given dose of stilbestrol than can occur in animals receiving adequate amounts of thiamine but limited in intake of basal ration so that body weight is not significantly different from that found in the deficient animals (except at the 400 μg. dose of stilbestrol in this case).

Evaluation of the Synthetic Diet.—It can be seen from Table 5 that both breeds of chicks grow as well on the complete synthetic diet as on a good commercial mash. The Sex-linked Cross breed, however, gives a signif-

TABLE 6. COMPARISON OF OVIDUCT RESPONSES TO STILBESTROL IN CHICKS FED A COMMERCIAL MASH PLUS VITAMIN B_{12} OR THE COMPLETE SYNTHETIC DIET PLUS VITAMIN B_{12}*

Breed	Stilbestrol total dose μg.	Complete synthetic diet +B_{12}			Commercial mash +B_{12}			Mean body weight difference		Mean oviduct weight difference	
		No. of chicks	Mean body weight gm. ± S.E.	Mean oviduct weight mg. ± S.E.	No. of chicks	Mean body weight gm. ± S.E.	Mean oviduct weight mg. ± S.E.	t	(P)	t	(P)
W.L.	0	12	108 ± 7	23 ± 2	14	108 ± 2	24 ± 1	0.0			
	3200	12	116 ± 9	799 ± 17	14	113 ± 3	891 ± 40	0.3		0.6	
S.L.	0	14	131 ± 7	29 ± 2	12	147 ± 5	30 ± 2	2.0	(0.05)		
	3200	14	151 ± 4	885 ± 39	13	152 ± 5	1036 ± 49	0.2		2.4	(0.05–0.02)

* Experiment 54; 18 days on diet.

icantly greater response to stilbestrol stimulation on the synthetic diet. Addition of vitamin B_{12} to either diet does not change its nutritive value for the White Leghorns (Table 6) with respect to body weight or oviduct response to estrogen. (In all cases $P > 0.05$.) For the Sex-linked Cross breed supplementation with vitamin B_{12} (Tables 5 and 6) results in an almost significant body weight increase in the stilbestrol-treated birds on the synthetic diet ($P = 0.1$-0.05) and a definitely significant body weight increase in the stilbestrol-treated birds on the commercial mash ($P = 0.01$). The relationship of vitamin B_{12} to stilbestrol action cannot be interpreted on the basis of the data presented here. All that can be said is that there is significantly greater response to a 3.2 mg. dose of stilbestrol in the Sex-linked Cross breed on commercial mash when it is supplemented with vitamin B_{12} ($P = 0.05$), but that supplementation of the synthetic diet with vitamin B_{12} significantly decreases the oviduct response to this dose of stilbestrol ($P < 0.01$).

Inanition.—In six experiments in which the influence of inanition on the

TABLE 7. OVIDUCT RESPONSE TO STILBESTROL IN CHICKS SUFFERING FROM INANITION

Exp. No. (Breed)	Stilbestrol total dose μg.	Complete synthetic diet unlimited food intake			Complete synthetic diet limited food intake (inanition)			Mean body weight difference t	Mean oviduct weight difference t
		No. of chicks	Mean body weight gm. ± S.E.	Mean oviduct weight mg. ± S.E.	No. of chicks	Mean body weight gm. ± S.E.	Mean oviduct weight mg. ± S.E.		
30 (S.L.)	1250	22	108 ± 3	457 ± 20	22	70 ± 2	481 ± 21	10.3	0.8
32 (S.L.)	1250	12	108 ± 4	472 ± 34	14	63 ± 2	367 ± 15	9.7	2.8
35 (S.L.)	1250	24	91 ± 10	426 ± 7	20	67 ± 7	363 ± 9	9.6	2.6
46 (S.L.)	100	12	114 ± 4	84 ± 6	9	60 ± 7	62 ± 5	11.3	2.6
	200	12	125 ± 6	163 ± 10	10	60 ± 2	127 ± 6	11.2	3.8
	400	10	129 ± 4	330 ± 11	7	61 ± 3	225 ± 6	18.4	8.1
	1600	11	124 ± 4	613 ± 34	8	62 ± 3	367 ± 24	12.2	7.8
48 (S.L.)	100	8	141 ± 5	136 ± 26	11	69 ± 3	57 ± 5	12.4	3.1
	400	10	131 ± 6	350 ± 29	14	76 ± 2	260 ± 16	8.7	2.7
	1600	10	144 ± 6	666 ± 41	14	79 ± 3	574 ± 27	9.3	1.9
50 (W.L.)	0	11	95 ± 8	24 ± 5	13	58 ± 3	16 ± 1	—	1.5
	1750	12	110 ± 13	609 ± 46	11	63 ± 2	459 ± 40	—	2.5

Days on diet, 16.

response of the chick oviduct to stilbestrol was studied it was observed that over a wide range of doses of stilbestrol, the animals suffering from inanition had oviduct weights significantly lower than those of animals supplied with the basal ration in unlimited amounts, and that, in general, the more severe the inanition, the lower the response to stilbestrol (Table 7).

Inanition was produced by limiting the basal ration only, the vitamin supplement having been given in the same amount to both groups, so that barring differences in ability to absorb and utilize the vitamins, deficiencies of the vitamins included in the oral supplement have been excluded in the inanition state. The limitation of the basal ration was adjusted to parallel the gradual decrease in food intake occurring as vitamin deficiencies developed in the experimental groups. Any quantitative inadequacy of the various components which resulted from this restriction of a qualitatively adequate diet therefore occurred in both the experimental and control groups.

In all instances except two, the responses of animals suffering from inanition were significantly lower than those of the normal controls. In one case, Experiment 30, the responses were the same. In the second case, Experiment 48, at 1600 μg. stilbestrol, the reduction in response was almost statistically significant ($P = 0.1\text{-}0.05$).

Reports in the literature indicate that rats suffering from inanition fail to inactivate estrone and α-estradiol absorbed from pellets implanted in the spleen (Drill and Pfeiffer, 1946; Jailer, 1948). If a state of inanition in the chick also results in decreased inactivation of stilbestrol injected subcutaneously, then in our chicks the responsiveness of the oviduct must be even more greatly depressed by inanition than the data reveal.

DISCUSSION

A vitamin deficiency is accompanied by a state of inanition which involves not only a decrease in general body growth but also a nonspecific reduced sensitivity of the chick oviduct to estrogen stimulation. Superimposed on this general debility is the specific relationship of the vitamin to the mechanism of action of the hormone. Conceivably, a vitamin may be either a part of an enzyme system involved in inactivation of the estrogen, or essential to an enzyme system through which the estrogen exerts its biological function, or both.

Elimination of the inanition aspect of a vitamin deficiency from consideration would aid in clarifying the role of an individual vitamin in the mechanism of action of stilbestrol. This has been attempted through the use, as controls, of animals in which inanition was induced by limitation of the basal ration but not of intake of the B-complex vitamins. Such a procedure seems justifiable since it was found that the limitation of food

even in the presence of adequate vitamin supplements produced not only smaller animals but a decreased sensitivity of the oviduct to stilbestrol (Table 7). As shown in Table 8, at each of the dose levels of stilbestrol studied, a positive correlation was found between the body weight and the oviduct weight; for the dose levels of 100, 200, 400, and 1600 μg., respectively, correlation coefficients of 0.560, 0.364, 0.663, and 0.521 were calculated. In each instance the P value was less than 0.001. The possibility therefore exists that in any vitamin deficient animal showing a stunting of general body growth, the oviduct responsiveness may be decreased "nonspecifically" by the inanition suffered by the animal apart from any specific change attributable to the particular vitamin in question.

TABLE 8. THE CORRELATION BETWEEN BODY WEIGHT AND OVIDUCT RESPONSE TO STILBESTROL IN CHICKS*

Stilbestrol total dose μg.	No. of chicks	Mean body weight gm. (Range)	Mean oviduct weight gm. (Range)	Correlation coefficient r	Probability P
100	77	98 (48–162)	78 (33–250)	0.560	<0.001
200	64	101 (50–160)	140 (80–270)	0.364	<0.001
400	81	101 (50–158)	278 (126–490)	0.663	<0.001
1600	82	103 (49–178)	524 (256–841)	0.521	<0.001

* The Sex-linked Cross breed of chicks was used. All animals received the same supplement of vitamins. Stunted animals were limited in intake of basal synthetic diet. All chicks were autopsied at 18 days of age.

In the section in which experimental results were presented, it was shown that in comparison to normal animals, chicks deficient in PGA, nicotinic acid, riboflavin, or thiamine exhibited certain characteristic responses to stilbestrol stimulation. A deficiency of PGA markedly decreases the response to stilbestrol. When there is a deficiency of nicotinic acid, stilbestrol elicits a greater response at lower doses and an equal or smaller response at higher doses than is expected in normal animals, depending on the breed. A deficiency of riboflavin limits the response to stilbestrol while a thiamine deficiency appears to have no effect on it.

When the common factor of inanition is taken into consideration by comparing the oviduct responses of vitamin deficient chicks to those of inanition (weight) controls (Table 9), somewhat different interpretations of the relationships of the vitamins to the utilization of stilbestrol becomes possible. In PGA-deficient chicks there is, at all dose levels of stilbestrol studied, an inability of the oviduct to respond to stilbestrol that cannot

be accounted for simply on the basis of inanition. PGA may, therefore, possibly be involved in the enzyme system through which stilbestrol exerts its effect at the level of the end organ itself.

In contrast to those suffering from a PGA-deficiency, animals deficient in riboflavin show a decreased oviduct sensitivity only at the 100 μg. dose of stilbestrol when compared to inanition (weight) controls. Comparison of riboflavin deficient animals to inanition controls indicates a relative sensitivity of 101% ± 14% of the latter group in terms of the former at the dose levels of stilbestrol used. Thus, no difference in oviduct response could be demonstrated. Since the inanition factor has been evaluated this similarity in response may be due to the fact that either no change in

TABLE 9. OVIDUCT RESPONSE TO STILBESTROL IN VITAMIN DEFICIENT CHICKS COMPARED TO THAT OF INANITION CONTROL CHICKS*

Vitamin deficiency	Stilbestrol total dose μg.	Vitamin deficient chicks			Inanition control chicks			Oviduct weight P
		No. of chicks	Mean body weight gm. ± S.E.	Mean oviduct weight mg. ± S.E.	No. of chicks	Mean body weight gm. ± S.E.	Mean oviduct weight mg. ± S.E.	
Riboflavin	100	14	60 ± 2	47 ± 4	15	61 ± 2	61 ± 4	0.02
	200	10	64 ± 3	107 ± 15	13	65 ± 3	125 ± 5	0.30
	400	10	55 ± 2	245 ± 22	8	60 ± 2	236 ± 8	>0.5
	1600	11	55 ± 1	433 ± 37	8	59 ± 2	376 ± 31	>0.5
Thiamine	100	14	63 ± 2	86 ± 7	12	60 ± 2	63 ± 5	<0.01
	400	14	65 ± 1	346 ± 15	14	65 ± 2	236 ± 9	<0.01
	1600	11	60 ± 3	506 ± 29	12	63 ± 2	404 ± 25	<0.01
PGA	100	15	68 ± 2	36 ± 2	15	67 ± 2	62 ± 2	<0.001
	200	14	80 ± 3	41 ± 4	15	80 ± 3	124 ± 7	<0.001
	400	10	80 ± 4	77 ± 5	17	75 ± 2	246 ± 20	<0.001
	1600	10	77 ± 3	95 ± 11	15	80 ± 2	532 ± 37	<0.001
Nicotinic Acid	100	12	82 ± 4	105 ± 9	13	82 ± 2	70 ± 6	<0.01
	200	12	80 ± 4	187 ± 16	13	78 ± 3	122 ± 16	0.01
	400	12	70 ± 3	243 ± 10	20	70 ± 2	240 ± 10	>0.50
	1600	14	76 ± 4	371 ± 22	17	76 ± 2	543 ± 24	<0.01

* Sex-linked Cross breed.

stilbestrol inactivation and no change in oviduct responsiveness occurs, or that both are influenced, the algebraic sum of the changes being zero.

Thiamine-deficient chicks illustrate a third type of response. In the absence of this vitamin, increased oviduct response was obtained at dose levels of 100, 400, and 1600 μg. of stilbestrol. Over the entire range of doses of hormone, the thiamine deficient chicks had an oviduct response 174% ± 13% of that found for the inanition weight controls. Thiamine deficiency appears to interfere with stilbestrol inactivation and thus, to produce an increased oviduct response for a given dose of estrogen.

Of particular interest in nicotinic acid deficiency is the increased response for the vitamin deficient animals, as compared to inanition controls, at the lower dosed of 100 and 200 μg. of stilbestrol and a reversal of response at the highest dose level of 1600 μg. At the intermediate level of 400 μg. of stilbestrol identical oviduct responses occurred. The difference in respon-

siveness between vitamin deficient animals and the inanition weight control animals was analyzed using the statistical design of Bliss (1944) at the 100 and 200 μg. levels. Since the dosages of stilbestrol were identical for the two experimental conditions the differences in oviduct response can be taken as a measure of responsiveness. It was found that the nicotinic acid deficient animals gave an increased response of $169\% \pm 23\%$. The slopes of the two lines were not significantly different; t equals 1.351. This agrees with the findings of De Meio *et al.* (1948) and Coppedge *et al.* (1950) who have reported, on the basis of studies *in vitro*, that diphosphopyridine nucleotide is involved in the inactivation of α-estradiol.

The fact that a decreased response to stilbestrol was found at the highest dose level of 1600 μg. may be due to a second function of nicotinic acid and the particular conditions of the experiment. Thus, if nicotinic acid were required for both the inactivation of the stilbestrol and the stimulation of the oviduct a partial deficiency of the vitamin might interfere more seriously with the inactivation than with the requirement for oviduct growth. At the higher dose level of stilbestrol, however, the quantity of nicotinic acid present for oviduct growth may be the limiting factor. In the experiments reported it is not unlikely that a small but very limited amount of nicotinic acid is present. We may conclude, therefore, that nicotinic acid probably is an essential component of the system which inactivates stilbestrol and may also be required for the optimal growth of the oviduct in response to estrogen stimulation.

SUMMARY

The inanition which accompanies a state of vitamin deficiency is responsible for a nonspecific, reduced sensitivity of the oviduct to the action of estrogen. When this reduced sensitivity is taken into consideration it is found that estrogen exerts a greater-than-expected stimulation in thiamine-deficient pullets, probably explainable on the basis of greater availability of estrogen because of failure of inactivating mechanisms. In riboflavin-deficient pullets the reduced response to estrogen may be accounted for, superficially at least, on the basis of inanition alone but may be the algebraic sum of numerous factors such as reduced inactivation of estrogen and decreased functioning of the mechanisms for tissue proliferation. In nicotinic acid-deficient pullets low doses of estrogen can exert a greater-than-expected influence on oviduct growth but high doses are less effective than in inanition controls. A deficiency of pteroylglutamic acid (PGA), however, results in a severe limitation of response to estrogen at all dose levels. This confirms Hertz's observations (1945). The implication is that PGA is essential to the mechanism of action of estrogen in promoting tissue proliferation in the chick oviduct at the level of the end organ itself.

The naturally occurring estrogen, estrone, is similar to the synthetic stilbestrol in that it also does not effect an optimal stimulation of the oviduct in chicks deficient in PGA.

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COMPARISON OF SENSITIVITIES OF THE IMMATURE AND PREGNANT MOUSE FOR ESTIMATION OF GONADOTROPIN

AARON J. LADMAN AND MEREDITH N. RUNNER

Indiana University, Department of Anatomy and Roscoe B. Jackson Memorial Laboratory[1]

BOTH increase in weight of reproductive organs in the immature mouse and ovulation in the pregnant mouse have been demonstrated as responses to gonadotropins. These responses have been and still are used as tests for potency of gonadotropic substances. As yet, however, the relative sensitivities of assays using the immature and pregnant mouse apparently have not been reported. A comparison of these responses was undertaken therefore, using as a source of gonadotropin the unfractionated, dried pituitary glands collected from mice shortly after parturition. Observations for parturition were made in a manner similar to that used in an earlier study (Runner and Ladman, 1950). A brief account of the data on comparative sensitivities of the 2 assays has been reported previously (Ladman, 1949).

The immature female mouse was shown by Aschheim and Zondek (1928) to respond to the gonadotropin in urine of pregnant women. Other authors, namely, Hamburger and Pedersen-Bjergaard (1937), Levin and Tyndale (1937), and Greep, Fevold and Hisaw (1936) have shown the action of gonadotropin in immature mice and rats to be on the ovary directly, and upon the uterus and seminal vesicle indirectly. Irrespective of the manner of action, gonadotropins result in a measurable increase in the weights of the above mentioned organs. This change in weight of reproductive organs due to the administration of gonadotropin has been studied extensively, and bioassays have evolved which incorporate their responses.

The ovulatory response to gonadotropins by mice was originally reported by Smith and Engle (1927) after having transplanted pituitary glands. Susbequently, this response was adopted by Hill, Parkes and White (1934), Rowlands (1935), and Friedman (1939), using rabbits and by Burdick *et al.* (1943), and Saunders (1947), using mice as an assay procedure for gonadotropins.

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METHODS AND MATERIALS

Donor and assay animals throughout these experiments were Swiss mice. They were fed a diet of Purina Fox Checkers supplemented weekly with rolled oats. For comparison of 2 assay methods, the employment of a commercially standardized pituitary preparation was considered to be the procedure of choice. However, our present and future interests are concerned with the estimation of gonadotropin in the female mouse, therefore, pituitary glands which were carefully collected from postpartum mice were utilized. Postpartum mice, from which pituitaries were assayed, were 6 to 10 months of age. While pregnant they were kept in individual cages and observed 6 times per day, every 4 hours, to obtain accurate data on the diurnal influence upon parturition and ovulation as reported earlier (Runner and Ladman, 1950). Pituitary glands were collected from 244 mice immediately after finding newborn young. Since the mice were observed every 4 hours, the glands were taken on an average of 2 hours after delivery. These pituitary glands, collected from animals delivering at all times of day and night, were pooled and stored in acetone in groups of 15 to 25 pituitaries and were regarded as being essentially similar in potency. This assumption was justified by the relative uniformity of consecutive tests. Assays were made as animals became available at which times groups of pituitaries were homogenized in acetone, dried in a vacuum dessicator, rehomogenized in physiological saline solution and injected as a fine suspension.

Immature assay animals, totaling 257, were selected to be 5.5 to 7.5 grams at the age of 16 days so that at autopsy, 5 days later, the body weights were between 7 and 10 grams. The animals for assays were injected with pituitary suspension according to the technique employed by Fiske (1941). The females were injected subcutaneously, the males intraperitoneally, twice daily for four days and autopsied on the morning of the 5th day, at 21 days of age. Controls received similarly scheduled injections of physiological saline. Uteri were dissected clear of surrounding connective tissue and fat, fluid was expressed from the lumina by blotting between filter paper, and the organs were then immediately weighed on a torsion balance. Ovaries and seminal vesicles, after being freed from the ovarian capsule and prostate gland respectively, were quickly weighed on the torsion balance.

Adult assay animals, 15 to 19 days pregnant, received a single injection of 0.5 cc. pituitary suspension intraperitoneally, and 18 hours later were examined for the presence of tubal ova. If an animal was not to be sacrificed, it was anesthetized; an incision was made in the dorsolateral body wall and the ovary and oviduct easily presented. Tubal ampullae containing ova were swollen and transparent and plainly showed their contents in situ when observed through a dissecting microscope (10×). Verification and counting of ova, when necessary, was accomplished by excising the ampullae, putting them in a drop of physiological saline and teasing them apart. As a result of this procedure, ova were extruded and easily counted. Since it had been shown (loc. cit.) that the mouse ovulates spontaneously 12 to 18 hours after delivery, valid results were obtainable in some instances when animals delivered between the time of injection and observation. Care was taken under these circumstances to use only animals that delivered within 10 hours prior to observation for tubal ova. On the basis of this earlier study, it could be assumed that ovulation within 10 hours after delivery was induced and not spontaneous.

The quantity of unfractionated pituitary given to the assay animals was expressed in terms of equivalents of whole pituitary glands and was adjusted by serial dilutions with physiological saline. End points or critical responses for the 2 assay procedures for this experiment, in keeping with data already presented in the literature, were regarded as the minimal amount of unfractionated pituitary gland, in terms of pituitary equivalents,

that (a) elicited a 100% increase in the organ-body weight ratio of the uteri, ovaries, and seminal vesicles in immature animals (Hamburger and Pedersen-Bjergaard, 1937; Levin and Tyndale, 1937; Greep, Fevold and Hisaw, 1936; Lloyd *et al.*, 1949) and (b) induced an ovulatory response in 50% of the pregnant assay animals (Hill, Parkes and White, 1934; Rowlands, 1935; Friedman, 1939; Burdick *et al.*, 1943; Saunders, 1947).

RESULTS

Changes in weights of organs in immature mice, as a result of stimulation by unfractionated pituitary glands, are expressed in this report (table 1, figure 1) as the per cent increase of organ weight per 100 gms. of body weight above that of the controls. This mode of treatment, in

TABLE 1. RESPONSE OF ORGANS OF IMMATURE MICE TO INJECTIONS OF DRIED PITUITARY

Indicator organ	Dosage in pituitary equivalents	Number of animals observed	Organ weight per 100 gms. of body weight ± standard error	Per cent increase
Uterus	0	41	88.8 ± 1.92	control
	½	18	85.4 —	0%
	1	16	109.8 ± 6.86	24%
	2	5	156.9 ± 29.38	77%
	4	5	351.2 ± 22.85	296%
Ovary	0	41	28.4 ± 1.06	control
	½	18	23.2 —	0%
	1	16	28.0 ± 1.10	0%
	2	8	33.6 ± 2.64	18%
	4	8	39.4 ± 2.55	39%
Seminal Vesicle	0	38	20.9 ± 0.96	control
	½	16	26.4 ± 1.30	26%
	1	11	27.5 ± 1.46	32%
	2	8	35.1 ± 1.96	68%
	4	8	46.5 ± 2.96	123%

contradistinction to reports which have utilized the per cent deviation from the absolute organ weights of controls, narrowed the range of variation in organ weights by compensating for what differences that did exist in body weight of the assay animals. Although the animals used in the present study had a relatively small range of weights, 3 grams at autopsy, prior investigations of this type have neither held to a correspondingly small range nor have they always compensated for differences in weight. Our experience reaffirms the position of Barlow and Sprague (1941) who stressed the importance of uniform weight as well as uniform age of animals used in bioassays.

Data obtained from estimation of potency of pituitary glands using immature animals for assay are given in table 1. It can be seen that the uterus gave the critical increase in response to the pituitary injections between the dosage levels of 2 and 4 pituitary equivalents, 77% and 296% respec-

tively. The ovaries, although they responded, did not give a critical increase in weight at the dosages used, 18% at the level of 2 pituitary equivalents and 39% at 4 pituitary equivalents. At all dosage levels, the seminal vesicles gave a positive increase with the critical changes occurring, as in the case of the uterus, between the levels of 2 and 4 pituitary equivalents, 68% and 123% respectively. The magnitude of increase of the seminal vesicles to 4 pituitary equivalents was inter-

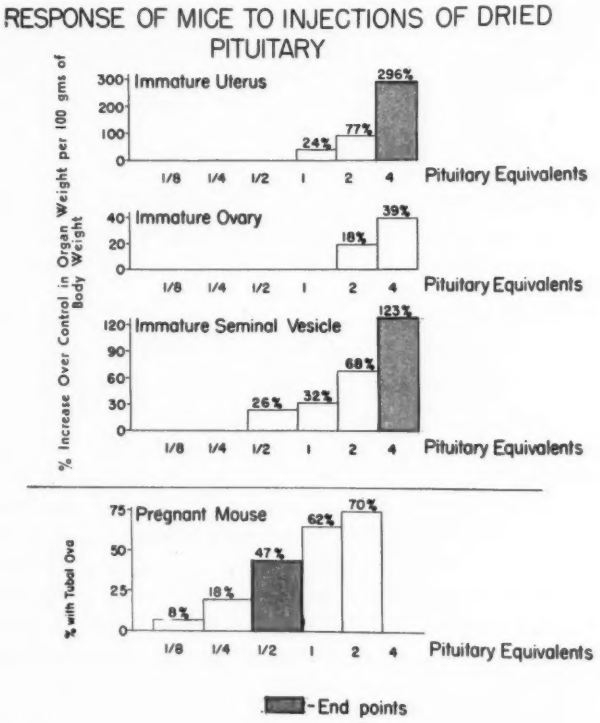

FIG. 1

mediate in response between that of the uterus and ovary. Information obtained concerning the per cent change of the above organs as compared to the controls is illustrated in figure 1. It is evident that the uterus gave a response about 7 times larger than the ovary at the critical dosage level of the former. In summary, then, critical changes for both uteri and seminal vesicles were first obtained at the level of approximately 4 pituitary equivalents. The ovarian responses, at the same dosage level were 39%; far short of the 100% increase which is required for significance according

to the criterion of Hamburger and Pedersen-Bjergaard (1937). Each pituitary gland therefore, contained about $\frac{1}{4}$ of the amount of gonadotropic hormone necessary to produce a critical response in the uterus and seminal vesicle.

Data indicating the response of pregnant mice to injections of unfractionated pituitary glands by the presence of induced ova are summarized in figure 1 and table 2. The end point, as defined earlier, was seen to be at approximately the level of $\frac{1}{2}$ pituitary equivalent, at which dosage, 9 out of 19 animals or 47%, had tubal ova. Since $\frac{1}{2}$ gland was sufficient to give a critical response, each whole pituitary, therefore, contained about 2 ovulatory units of gonadotropin.

TABLE 2. OVULATORY RESPONSE OF PREGNANT FEMALES TO INJECTIONS OF DRIED PITUITARY

Dosage in pituitary equivalents	Number of assay animals	Number induced to ovulate	Per cent ovulation
$\frac{1}{8}$	11	1	8%
$\frac{1}{4}$	17	3	18%
$\frac{1}{2}$	19	9	47%
1	13	8	62%
2	10	7	70%

Comparisons of reactions of the immature and pregnant assay animals are demonstrated in figure 1. The dosage, $\frac{1}{2}$ pituitary equivalent, which produced a critical ovulatory response in the pregnant female gave only a slight increase in organ-body weight ratio, 26%, on the part of the seminal vesicles. Twice this dosage, 1 pituitary equivalent, was necessary to produce an appreciable increase in the weight of the uterus and 4 times this dosage, 2 pituitary equivalents, had to be given before the ovary reacted positively at all. Both uteri and seminal vesicles gave critical increases in organ-body weight ratios at the dosage level of 4 pituitary equivalents—8 times that needed to produce a critical response in pregnant females.

DISCUSSION AND CONCLUSIONS

Responsiveness to gonadotropins on the part of the immature reproductive organs substantiate previously reported data obtained from a variety of gonadotropins in the hands of Hamburger and Pedersen-Bjergaard (1937); Levin and Tyndale (1937); Greep, Fevold and Hisaw (1936); Fiske (1941); Lloyd *et al.* (1949); Evans *et al.* (1940); D'Amour and D'Amour (1940); and Heller, Lauson and Sevringhaus (1938). There seems to be general agreement that the uterus, although indirectly affected by gonadotropins, is a sensitive organ for assay purposes.

Statistical treatment of the data for organ-body weight ratios was applied. Standard error of the difference between the experimental and

control groups indicated that, for the data obtained, there were statistically significant increases for the uterus, ovary and seminal vesicle at responses of 77%, 39% and 26% respectively. These values are considerably lower than the criterion of 100% increase that has been used heretofore. This finding may be attributed to the relatively uniform weights of organs in conjunction with compensation for differences in body weights. It is indicated that if, as was done here, assay animals are carefully selected on the basis of age and weight, critical end points for assays using immature animals can be revised downward from the 100% response and still retain an acceptable level of confidence.

It should be mentioned that the immature animals besides serving as a comparison to the ovulation test, also indicate the possible balance of FSH and LH in the pituitary suspensions which were assayed. Increase in weight of uterus and ovary has served in the past as a specific biological indication of FSH activity. Similarly, the increase in weight of the seminal vesicle has indicated luteinizing (LH) activity in a hormone preparation. It can be seen from figure 1, by the response of the seminal vesicle and absence of change in the uterus and ovary to the lower dosage levels of the pituitary suspension, that the pituitaries collected at parturition were relatively potent in LH. Realizing that these pituitary glands were recovered 2 hours after the animals had delivered a litter, it may be indicated that the surge of hormone (LH) which will induce the first postpartum ovulation, as postulated previously (Runner and Ladman, 1950), had not yet been released from the pituitary.

Ovulatory responses of pregnant mice following injections of gonadotropic hormones have demonstrated that this reaction can be used as a sensitive and convenient end point for assay purposes. This conclusion derived from the present data supports observations of Burdick *et al.* (1943) and Saunders (1947). Where circumstances permit its use, the pregnant mouse ovulation test would have at least 4 advantages over tests using the immature mouse for purposes of assaying gonadotropin. 1) It is faster; the desired information can be obtained within 18 hours. 2) A single injection suffices instead of making a series of injections over a 4 or 5 day period. 3) There is a more definitive end point, i.e. the presence or absence of tubal ova obviates interpretations based upon weight or color reaction which may show continuous gradations. 4) The pregnant mouse is more sensitive by about 8 times than the use of 100% increase in organ-body weight ratio of immature mice.

SUMMARY

Data obtained from immature and pregnant mice indicated that pituitaries of postpartum mice contained $\frac{1}{4}$ the amount of gonadotropin

required to produce a critical response in the uterus and seminal vesicle and twice the amount needed to elicit the critical response in the pregnant mouse. It has been concluded therefore, that the pregnant mouse as a test animal is approximately 8 times more sensitive than conventional tests using the immature mouse.

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THE EFFECT OF ROENTGEN RAY IRRADIATION ON NORMAL, HYPOTHYROID AND HYPERTHYROID RATS[1]

THOMAS J. HALEY, SAMUEL MANN AND ANDREW H. DOWDY

School of Medicine, University of California at Los Angeles

BLOUNT and Smith (1949) reported that the feeding of thyroid extract to normal mice increased the mortality rate after roentgen ray irradiation while thiouracil feeding decreased the rate under similar circumstances. They concluded that the increased mortality was the result of an increased metabolic rate rather than an increased body temperature. Brophy and McEachern (1949) have shown that thyroxin not only increases the basal metabolic rate of the intact animal, but also the oxygen consumption of isolated tissues from such an animal. It appears, therefore, that the hyperthyroid animal is more susceptible to roentgen ray irradiation damage because of an increased oxygen consumption. The production of a hypothyroid animal with thiouracil is complicated by the fact that thiouracil has a potential sulfhydryl group in its molecule, and thus any protective effect observed may result by virtue of the mechanism of sulfhydryl group protection postulated by Patt *et al.* (1949) for the cysteine protection of roentgen ray irradiated rats.

We would like to report on the lethal effect of acute whole body roentgen ray irradiation in normal, hypothyroid and hyperthyroid rats where the hypothyroid state was produced surgically and was not complicated by the possible sulfhydryl protective action of thiouracil.

EXPERIMENTAL

Normal male Wistar Strain rats weighing 142 to 250 gm. (average 192 gm.) were thyroparathyroidectomized under ether anesthesia according to the procedure of Farris and Griffith (1949). Throughout the entire experimental period these animals received 0.1 per cent of calcium chloride in their drinking water. Both the normal and hypothyroid animals were weighed weekly. When the latter animals reached a weight plateau, the animals were separated into randomized groups of 20 animals each and injected subcutaneously with 0.1 mg./animal/day of Squibb thyroxine or Chilcott injectable thyroglobulin for five days pre-irradiation. The dose of the latter material was equivalent to the thyroxine dosage based upon metabolic response (Kroc, 1950). The stimulant

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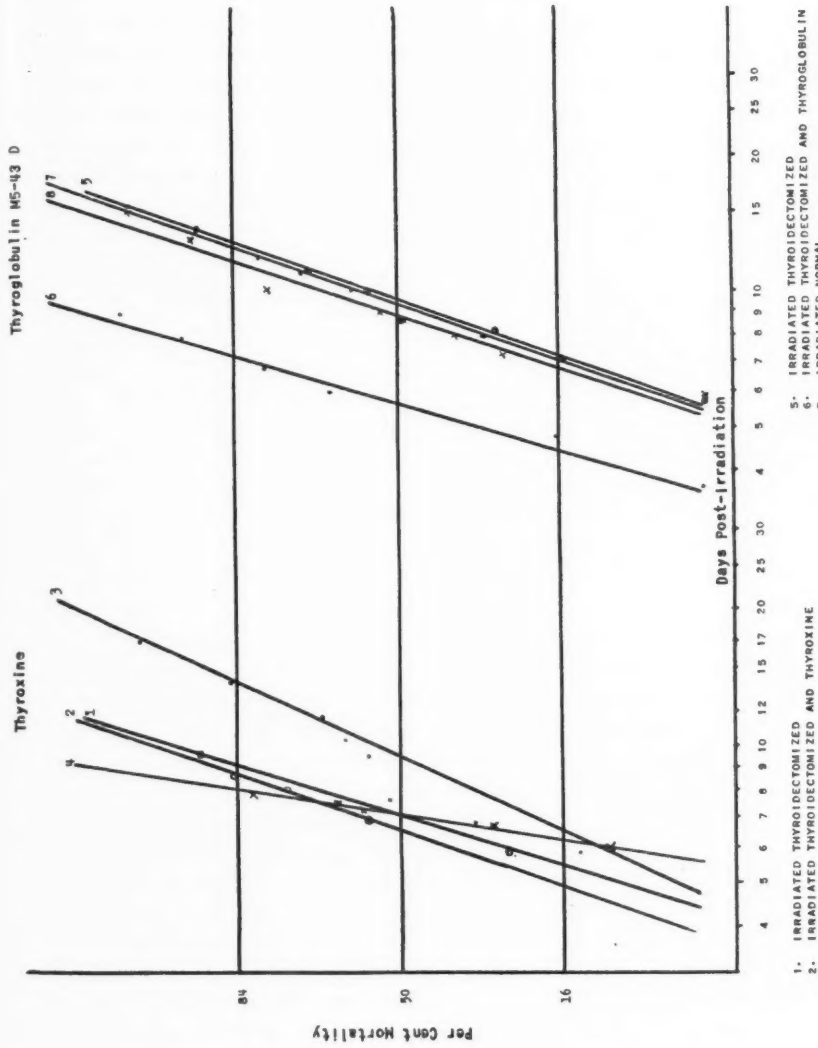
effect of the calorigenic agents made the medicated hypothyroid animals more active than their unmedicated counterparts. Increased irritability and weight losses indicated that the normal medicated animals were in a hyperthyroid state. Upon completion of the premedication period, the animals were subjected to 600 r acute whole body roentgen ray irradiation. The technical factors were: 250 KV; 15 MA; TSD 100 cm.; filters, 0.21 mm. Cu. inherent, 0.5 mm. Cu. parabolic and 1.0 mm. Al; HVL 1.85 mm. Cu.; size of field—total body; r/minute measured in air 9.6 to 9.66. Uniformity of dosage was insured by rotating the radiation cage during treatment. The 250 KV Picker Industrial Unit used was calibrated before each experiment with a Victoreen Thimble r meter. All the animals were maintained on their ordinary diet after irradiation. Autopsies were performed upon all the animals that died during the thirty day experimental period, and upon all survivors at the end of that time. The usual signs of irradiation damage (diarrhea, bloody stools, anorexia, petechial hemorrhages, pale mucous membranes, etc.) were observed in all irradiated animals. Gross examination of the thyroparathyroidectomized animals revealed no remaining thyroid tissue. Non-irradiated control groups of twenty animals each (normals, thyroxine or thyroglobulin medicated normals and thyroidectomized) were observed for thirty days.

RESULTS

The various non-irradiated control groups had no fatalities throughout the thirty day period. The results of our experiments on the other groups are shown in Graph 1. The thirty day total mortality in the irradiated groups varied between 85 and 100 per cent but the differences between groups were not significant. The mortality was due to the irradiation and not to the medication or the surgery, because there were no fatalities in the medicated or surgical controls during or after the experime tal period.

Inasmuch as there were observable differences in the slopes of the curves and the ET_{50} days in Graph 1, the data were analyzed statistically by the method of Litchfield (1949). This procedure showed that the slopes of the majority of the curves did not differ significantly and thus these curves were essentially parallel. However, the values of the slope ratios obtained by the comparison of curves 3 to 4, 2 to 4 and 4 to 8 exceeded their respective f_{SR} values showing that these curves deviated significantly from parallelism. Comparison of curves 3 to 4 and 2 to 4 indicates that thyroxine premedication significantly increases the susceptibility of normal and hypothyroid animals to damage from roentgen ray irradiation. Comparison of curves 4 to 8 shows that thyroxine has a more pronounced effect on roentgen ray irradiation damage in normal animals than an equivalent dose of thyroglobulin.

Only when the f_{SR} is greater than the reaction time ratio value is there any significant difference in the reaction time of the various groups. Thyroxine significantly accelerated roentgen ray irradiation mortality in the normal animls, whereas thyroglobulin did not (curves 3 to 4 and 7 to 8).


Thyroglobulin M5-43 D
Thyroxine
Per Cent Mortality
84
50
16
Days Post-Irradiation
4 5 6 7 8 9 10 12 15 17 20 25 30 4 5 6 7 8 9 10 15 20 25 30
1- IRRADIATED THYROIDECTOMIZED
2- IRRADIATED THYROIDECTOMIZED AND THYROXINE
3- IRRADIATED NORMAL
4- IRRADIATED NORMAL AND THYROXINE
5- IRRADIATED THYROIDECTOMIZED
6- IRRADIATED THYROIDECTOMIZED AND THYROGLOBULIN
7- IRRADIATED NORMAL
8- IRRADIATED NORMAL AND THYROGLOBULIN

GRAPH 1. Effect of roentgen ray irradiation on normal, hypothyroid and hyperthyroid rats.

Comparison of curves 1 to 3 and 5 to 7 indicates that the hypothyroid state may or may not increase the rate of roentgen ray irradiation mortality. However, this may not be really significant because comparison of curves 1 to 5 shows that the hypothyroid animals were more susceptible to irradiation damage in the first experiment. Furthermore, the hypothyroid animals in the second experiment were more responsive to the calorigenic agent (curves 5 to 6 and 6 to 8) because thyroglobulin had a greater effect on the rate of roentgen ray irradiation mortality than thyroxine. The susceptibility of hyperthyroid animals to roentgen ray irradiation damage was more pronounced when thyroxine was used to produce the hyperthyroid state (curves 4 to 8). No significant differences were detected in the response of the normal animals after roentgen ray irradiation, although the second experiment was performed nine months after the first one.

DISCUSSION

From these results as well as those of Blount and Smith (1949) it is evident that the hyperthyroid state is not conducive to the survival of roentgen ray irradiated animals. Surgical hypothyroidism does not afford even the small degree of portection observed with hypothyroidism from thiouracil. Possibly the concomitant parathyroidectomy may have produced these divergent results, but this would attribute a physiological property to parathyroid hormone other than the regulation of calcium and phosphorus metabolism. Insofar as these elements are concerned, the hypothyroid animals had sufficient of both, and the absence of tetany shows the animals were in calcium balance. It is more probable that the susceptibility of the normal, hypothyroid and hyperthyroid animals to roentgen ray irradiation damage is related to blood oxygen saturation and/or oxygen utilization by the various body tissues and to the circulation time of the blood. Zondek (1936) has reported that for the same oxygen tension (55 mmHg), the per cent oxygen saturation of the blood is approximately the same in normals and hypothroidism (80-85%), whereas it is much lower in hyperthyroidism (65%). However, increased utilization of, and greater tissue avidity for, oxygen would make the hyperthyroid animal more susceptible to radiochemical intermediates (peroxides) formed by ionizing radiation. The increased circulation time would also produce a faster body distribution of these materials in the hyperthyroid animal. These same principles would explain the increased susceptibility of the thyroxine or thyroglobulin medicated hypothyroid animal to roentgen ray irradiation damage. Similarly, the slower mortality rate in the hypothyroid and normal animals could be explained on a slower rate of distribution of the radio-chemical intermediates. It is possible that one

other factor, dissociability of oxyhemoglobin, may play a part in the radiation mortality rate while not influencing total mortality. Dissociability of oxyhemoglobin is increased in hyperthyroidism and decreased in hypothyroidism (Zondek, 1936). However, inasmuch as our results with normal and hypothyroid animals were equivocal, it is probable that oxyhemoglobin dissociation plays no part in the rate of or total mortality seen. It is more probable that the amount of free oxygen present determines the extent of roentgen ray irradiation damage. This would be in accord with the observations of Dowdy, *et al.* (1950) that roentgen ray irradiation damage is in part the result of radiochemical reactions involving free oxygen.

SUMMARY

Thyroparathyroidectomy does not significantly increase the survival time nor decrease the total mortality of roentgen ray irradiated rats. Hyperthyroidism produced by the subcutaneous injection of thyroxine into rats causes a significant increase in mortality rate as compared to normal and hypothyroid animals, even though there are no significant differences in total mortality. Under the same conditions thyroglobulin does not produce any significant difference in the rate of mortality. Thyroxine and thyroglobulin premedication of hypothyroid rats decreases their survival time but not their total mortality as compared to nonmedicated hypothyroid and normal animals.

Acknowledgment

We wish to express our thanks to Dr. Robert Kroc of Chilcott Laboratories, Division of The Maltine Company, for supplying us with the injectable thyroglobulin M5-43 D and the data concerning its metabolic activity in terms of thyroxine.

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SYNERGISM BETWEEN PITUITARY FOLLICLE STIMULATING HORMONE (FSH) AND HUMAN CHORIONIC GONADOTROPIN (HCG)[1]

MIRIAM E. SIMPSON, CHOH HAO LI AND HERBERT M. EVANS

From the Institute of Experimental Biology and the Division of Anatomy, University of California, Berkeley

IT HAS been known for years that the gonadotropic action in female rats of pituitary follicle stimulating hormone is augmented by simultaneous administration of interstitial cell stimulating substances, such as pituitary ICSH (LH, Fevold *et al.*, 1931) or human chorionic gonadotropin (Evans *et al.*, 1931). As FSH was more completely purified the same synergic reaction continued to be demonstrable. Some question arose, however, as to the degree of limitation of FSH effects in the complete absence of ICSH. It has become widely accepted that FSH alone cannot produce large follicles capable of producing enough estrogen to bring animals into estrus. As regards the phenomenon of synergism in the male it was reported (Evans *et al.*, 1934, 1937; Greep *et al.*, 1936) that the two types of hormone acted synergically also in the male. When FSH was purified sufficiently to meet the chemists' standards for physico-chemical purity: electrophoresis, diffusion and ultra-centrifugation (Li, Simpson, and Evans, 1949; Li, 1949) a reinvestigation of the biological properties of FSH was undertaken. As a part of this program the capacity of interstitial stimulating substances to act synergistically with FSH is being reexamined in both male and female rats. HCG[2] has been used in the present study.

FEMALE RATS

EXPERIMENTAL PROCEDURE

Female rats were hypophysectomized when 26 days of age and 7 days later were injected once daily for 3 days with various levels of FSH and HCG, given separately or together after in vitro combination. All injections were given subcutaneously. Dosages are expressed in terms of total dose administered over the 3 day period. Autopsy was performed 24 hours after the last injection. At autopsy ovaries were examined under a binocular microscope, at 10 times magnification, for follicular development, vascularity,

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[2] "Antophysin," Winthrop.

and corpus luteum formation. Ovaries were weighed and fixed, and histologic sections were later examined. The uterus was similarly inspected for size, thickness of wall, vascularity and estrous distension, then was drained and weighed.

RESULTS

TABLE 1. SYNERGISM BETWEEN FSH AND HCG IN HYPOPHYSECTOMIZED IMMATURE FEMALE RATS
(Subcutaneous Injection, 3 days)

Total dose		No. of rats	Ovaries wt.	Ovarian histology		Uterus	
FSH	HCG			Follicles	Interstitial cells	Wt.	Description
mg.	mg.		mg.			mg.	
1.000	—	3	25	lF*	Deficient	53	medium
0.500	—	6	23	m-mlF	Deficient	37	small
0.250	—	9	13	mF	Deficient	24	small
0.125	—	6	9	s-smF	Deficient	26	small
—	0.150	9	12	sF	Partial repair	30	small
—	0.050	16	9	sF	Deficient	24	small
—	0.010	4	5	sF	Deficient	22	small
1.000	0.050	3	102	m-lF, Cl	Repair	93	ml, fluid
0.500	0.050	6	51	lF	Partial repair	100	estrous
0.250	0.050	9	33	m-lF	Deficient	83	estrous
0.250	0.010	4	18	m-mlF	Deficient	64	estrous
0.125	0.150	6	18	sF	Repair	39	small
Hypophysectomized controls		196	10	sF	Deficient	24	small

* Abbreviations used are as follows:
s, m, l: small, medium, large
F: follicles
CL: corpora lutea

On injection of FSH alone, minimal stimulation of follicles was detected microscopically at doses of 0.100 to 0.125 mg.[3] Ten times this dose caused the development of large follicles without repair of the deficient interstitial cells. At this higher level the uterus was increased in weight and there was some accumulation of fluid but not full estrous development.[4]

The preparation of human chorionic gonadotropin (HCG)[5] at the maximum dose injected in hypophysectomized female rats, 0.150 mg., had as its

[3] The MED of the freshly prepared FSH was 0.050 to 0.100 mg. When the preparation was stored frozen, this value increased to 0.100 mg., at which level it remained relatively constant.

[4] FSH alone did not cause corpus luteum formation when given subcutaneously at doses of 1–2 mg., but did so when intraperitoneally administered; at this very high level, no repair of interstitial cells occurred.

[5] The MED of the HCG preparation used, when injected intraperitoneally into immature normal female rats, was 0.050 mg.

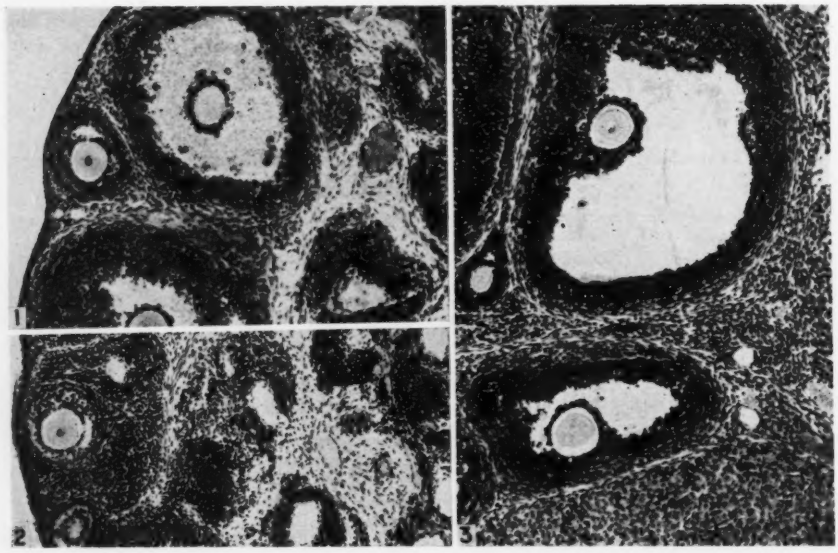

PLATE I

DESCRIPTION OF FIGURES

Control standards used in ovarian repair studies with pituitary and chorionic gonadotropins. Ovaries, H and E stain, ×92.

FIG. 1. Normal immature female rat, age 26 days.

FIG. 2. Hypophysectomized 26 day rat, 10 days postoperative.

FIG. 3. Normal adult female rat.

only effect partial repair of the deficient interstitial cells. The ovarian and uterine weights were barely increased by this dose.

Injection of FSH combined with HCG resulted in development of the reproductive system exceeding that anticipated from either component alone, both in degree of follicular development and in uterine development. At doses of FSH of 0.250 mg., augmentation of the follicular growth was best shown when combined with low doses of HCG (0.010 to 0.050 mg). In animals receiving such combinations the interstitial cells remained deficient or at most were only partially repaired. The synergism is illustrated in figures 4-6. Comparison with control standards may be made by consulting figures 1-3. Higher doses of FSH (such as 0.5 mg.) combined with 0.050 mg. HCG gave augmented follicular growth, still with only partially repaired interstitial cells, but combination with 0.150 mg. of HCG resulted in corpus luteum formation. Higher doses of FSH (1.0 mg.) required lower doses of HCG (0.050 mg.) for corpus luteum formation (Figures 7-9).

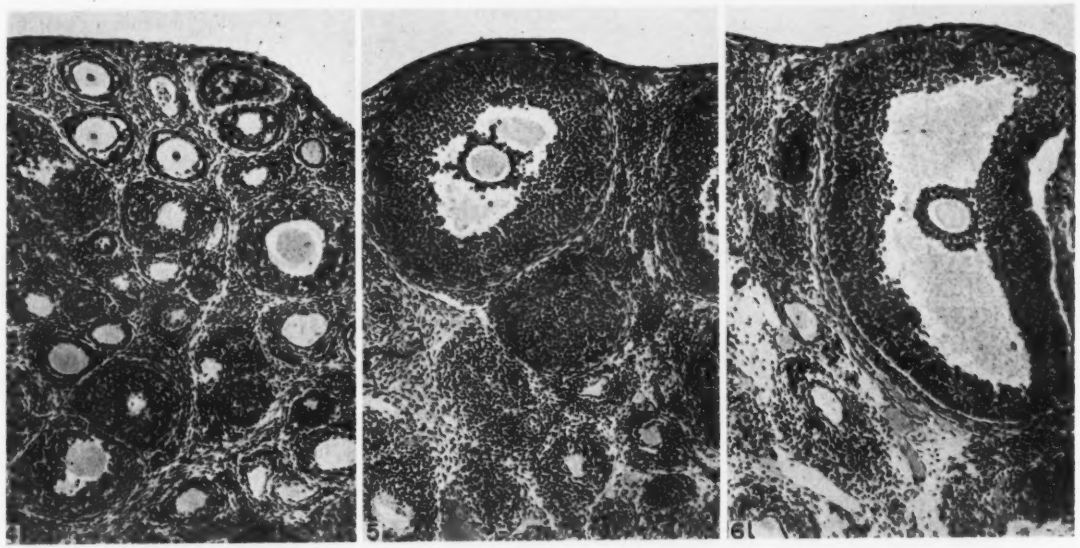

PLATE II

DESCRIPTION OF FIGURES

Synergistic effect of HCG on follicular development caused by FSH (Total dose in 3 subcutaneous injections, autopsy, 72 hours). Ovaries of hypophysectomized rats, H and E, ×92.

FIG. 4. HCG, 0.010 mg.

FIG. 5. FSH, 0.250 mg.

FIG. 6. Combination, as above.

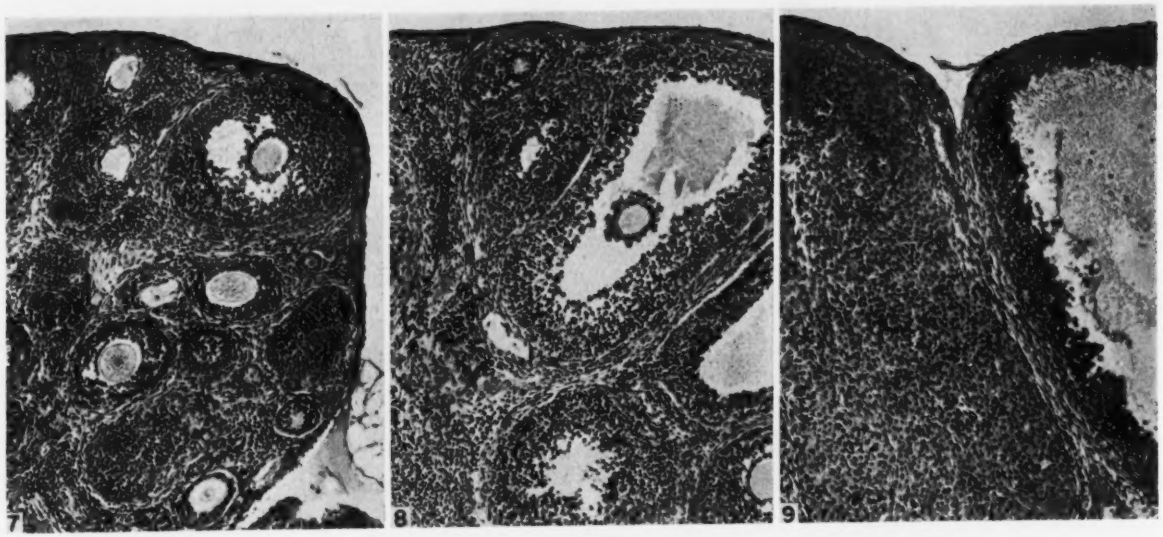

PLATE III

DESCRIPTION OF FIGURES

High doses of FSH require relatively low doses of HCG for corpus luteum formation. Ovaries of hypophysectomized rats, H and E, ×92.

FIG. 7. HCG, 0.050 mg.

FIG. 8. FSH, 1.0 mg.

FIG. 9. Combination, as above.

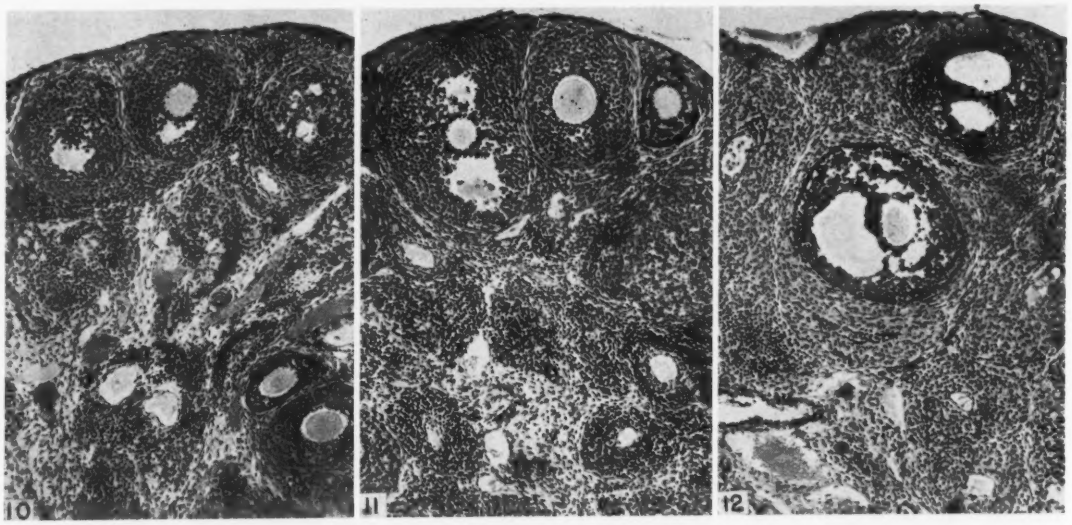

PLATE IV

DESCRIPTION OF FIGURES

Synergistic effect of FSH on interstitial tissue repair by HCG. Ovaries of hypophysectomized rats, H and E, ×92.

FIG. 10. HCG, 0.150 mg.

FIG. 11. FSH 0.125 mg.

FIG. 12. Combination, as above.

There was also evidence that the effectiveness of HCG in enlarging interstitial cells was somewhat increased by combination with FSH. A dose of HCG ineffective given alone, 0.050 mg. caused interstitial cell repair when combined with 0.5 to 1 mg. of FSH though these doses of FSH were ineffectual by themselves for repair of interstitial tissue (Figures 10-12).

MALE RATS

EXPERIMENTAL PROCEDURE

The effects of FSH and of HCG given alone or in combination in male rats were tested under the following experimental conditions. Male rats were hypophysectomized at 40 days of age and injections were begun the same day and continued daily for 15 consecutive days. Autopsies were performed 24 hours after the last injection, at which time the rats were 55 days old. At autopsy one testis was weighed, the other was fixed directly with epididymis attached, for histological examination. The contents of the other epididymis was examined fresh in warmed normal saline, for presence of motile spermatozoa. The epididymis was weighed in some instances before this examination. Seminal vesicles and ventral prostate were weighed.

This is the same type of experimental animal and the same procedure used in studies on the gametokinetic action of pituitary ICSH and of testosterone (Simpson, Li and Evans, 1944, Simpson and Evans, 1946). This test animal is peculiar in that hypophysectomy is performed when spermatids begin to appear in the testis, at the age of 40 days (Figure 13). At the termination of the experimental period (at 55 days of age) normal rats of this strain have begun spermatozoan formation and ripe spermatozoa are found in testicular tubules and epididymis (Figure 14). The testes of hypophysectomized, uninjected controls at the end of the 15 day experimental period (when 55 days of age) have regressed to a state where the tubules are lined only by spermatogonia, Sertoli cells and a few adherent spermatocytes (Figure 15); the interstitial cells are deficient, and the accessory organs of reproduction are atrophic.

As can be seen in Table 2, FSH exerted a marked effect on the testes of the hypophysectomized rats. Its action was detectable at daily doses of 0.050 to 0.100 mg. At these levels the tubules were maintained in slightly healthier condition than in the hypophysectomized control, more spermatocytes remaining adherent. At doses of 0.25 mg. though testis weight and tubular diameter regressed below that of the normal control of the age at operation, the tubules nevertheless contained differentiating spermatids with elongated, condensed, dark-staining nuclei. Even at these lower levels, doses of 0.10 to 0.25 mg., the testes occasionally showed further development (spermatozoa being formed in 8-30% of the rats). At a dose of 0.5 mg. the testes (and tubules) were about the size characteristic at operation, i.e., were maintained in size. Differentiation of the germinal elements was far more advanced, however, than in the 40 day normal, spermatozoa being present in most animals (80-100%), both in the testis and epididymis. A dose of FSH of 0.5 mg. was the minimal dose at which any effect

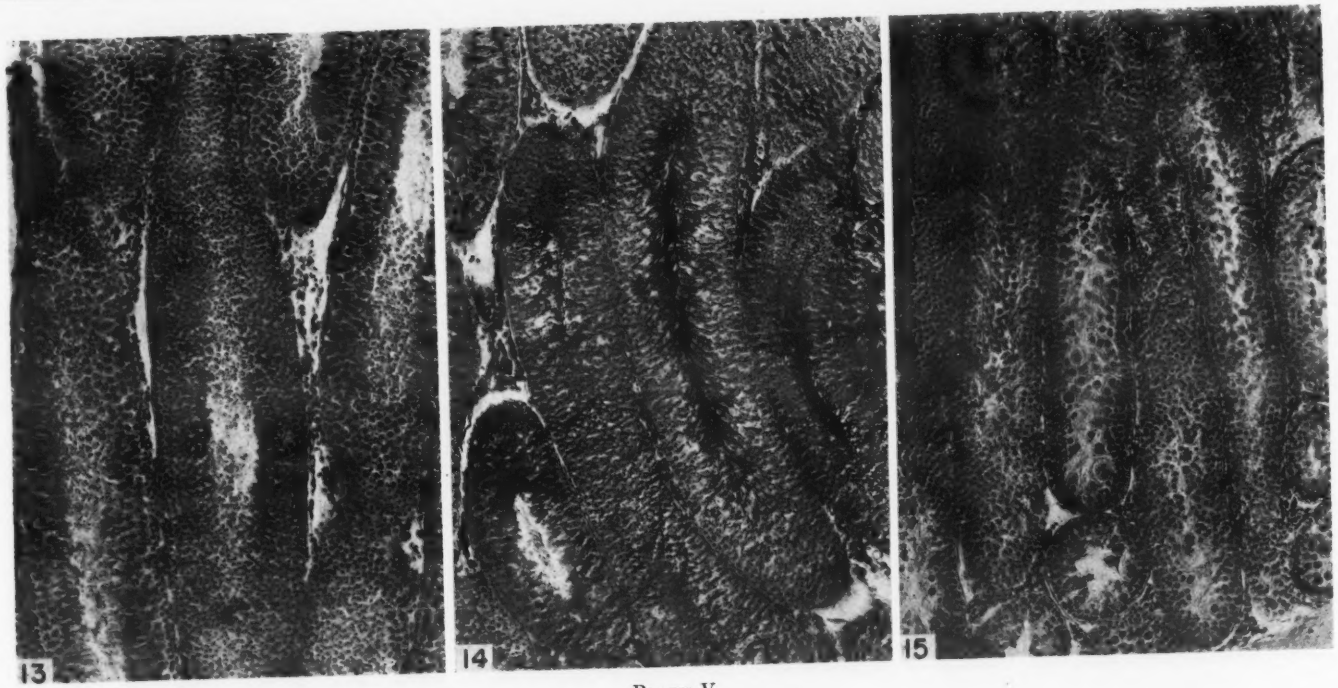

PLATE V

DESCRIPTION OF FIGURES

Control standards used in testicular maintenance studies in hypophysectomized 40 day male rats. Testes, iron hematoxylin, ×88.

FIG. 13. Normal 40 day rat. Spermatids are present.

FIG. 14. Normal 55 day rat. In the intervening 15 days, development has progressed to the formation of spermatozoa; young and mature forms are seen.

FIG. 15. Hypophysectomized 40 day rat, 15 days postoperative. The tubules have regressed, until only a few spermatocytes remain adherent.

RESULTS

TABLE 2. EFFECT ON REPRODUCTIVE SYSTEM OF HYPOPHYSECTOMIZED 40 DAY MALE RATS OF FSH AND HCG ALONE AND IN COMBINATION
(Maintenance test, 15 days, subc.)

Daily dose		No. of rats	No. with spermatozoa	Testes	Epi-didymis*	Seminal vesicles	Ventral prostate
FSH	HCG						
μg	μg			mg.	mg.	mg.	mg.
1000	—	4	4	1702		18	16
500	—	5	4	1188		16	12
250	—	10	3	773		19	12
100	—	20	2	622	39	14	13
50	—	12	0	643	34	16	10
—	25	5	5	1878		207	97
—	10	5	5	1803		138	77
—	5	10	8	1058		18	18
—	2.5	17	7	915	42	18	13
—	1.0	12	0	515	41	15	11
500	25	4	4	2212		407	178
	10	4	4	2296		392	103
	5	4	4	1914		133	52
250	25	5	5	2142		535	171
	10	4	4	2282		335	139
	5	8	8	1905		28	34
100	25	4	4	2149		222	123
	10	4	4	2094		102	75
	5	4	4	2026		61	53
	2.5	17	17	1497	107	19	20
	1.0	12	8	1116	53	19	14
50	2.5	12	10	1338	90	18	16
	1.0	12	3	838	75	16	11
Normal 55 da.		52	52	2355	182	255	119
Normal 40 da.		246	0	1345	74	42	63
Hypn. control		246	0	453	34	18	12

* Weights based on 5 rats each.

on the interstitial cells could be detected (partial maintenance[6]). At 1.0 mg. daily doses, motile spermatozoa were present in all injected rats. The interstitial cells were epithelioid (being intermediate in development between those of the 40 and the 55 day normal males) yet the accessory organs, seminal vesicles and ventral prostate, still showed no stimulation, being comparable in weight and structure to those of the hypophysectomized controls.

[6] The deficient interstitial cells of the hypophysectomized rat show small, darkly staining, round nuclei with clumped chromatin and scarcely any cytoplasmic body. At FSH doses of 0.5 mg. the nuclei were larger and more vesicular; the cytoplasmic body was distinguishable, though not collected around the nucleus as in an epithelial cell (epitholioid).

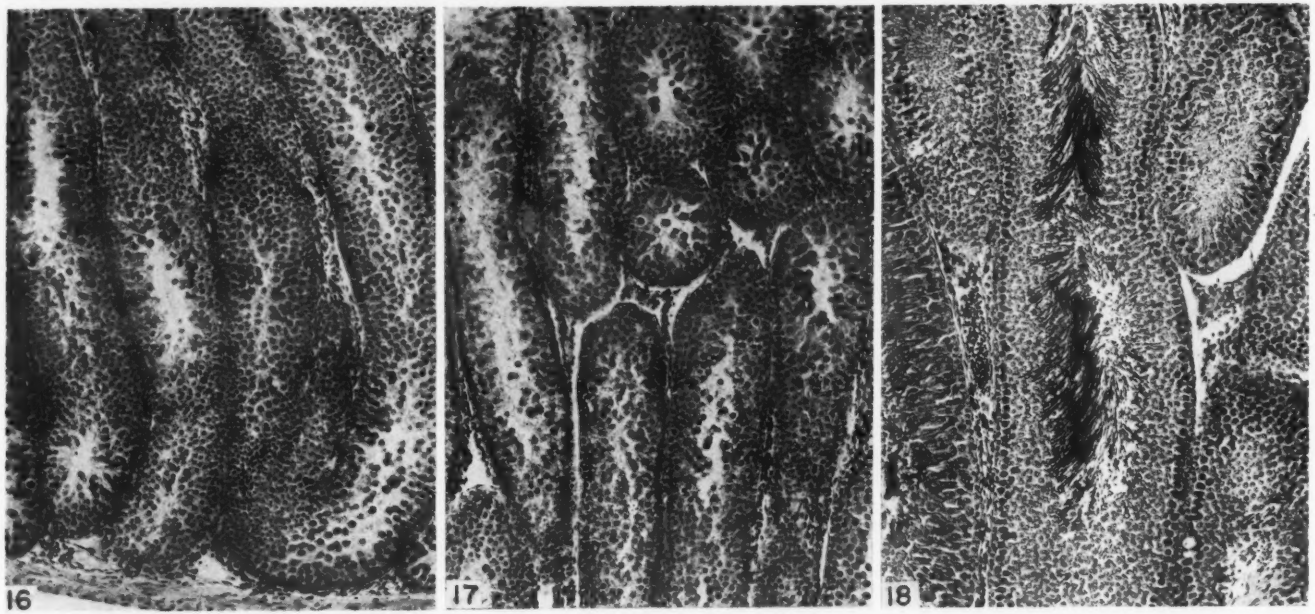

PLATE VI

DESCRIPTION OF FIGURES

Synergistic effect of low doses of FSH and HCG on the testicular tubules of hypophysectomized 40 day male rats. Testes, iron, hematoxylin, ×88.

FIG. 16. FSH, 0.1 mg. daily, 15 days, subc.

FIG. 17. HCG, 0.0025 mg. daily, 15 days, subc.

FIG. 18. Combination, as above.

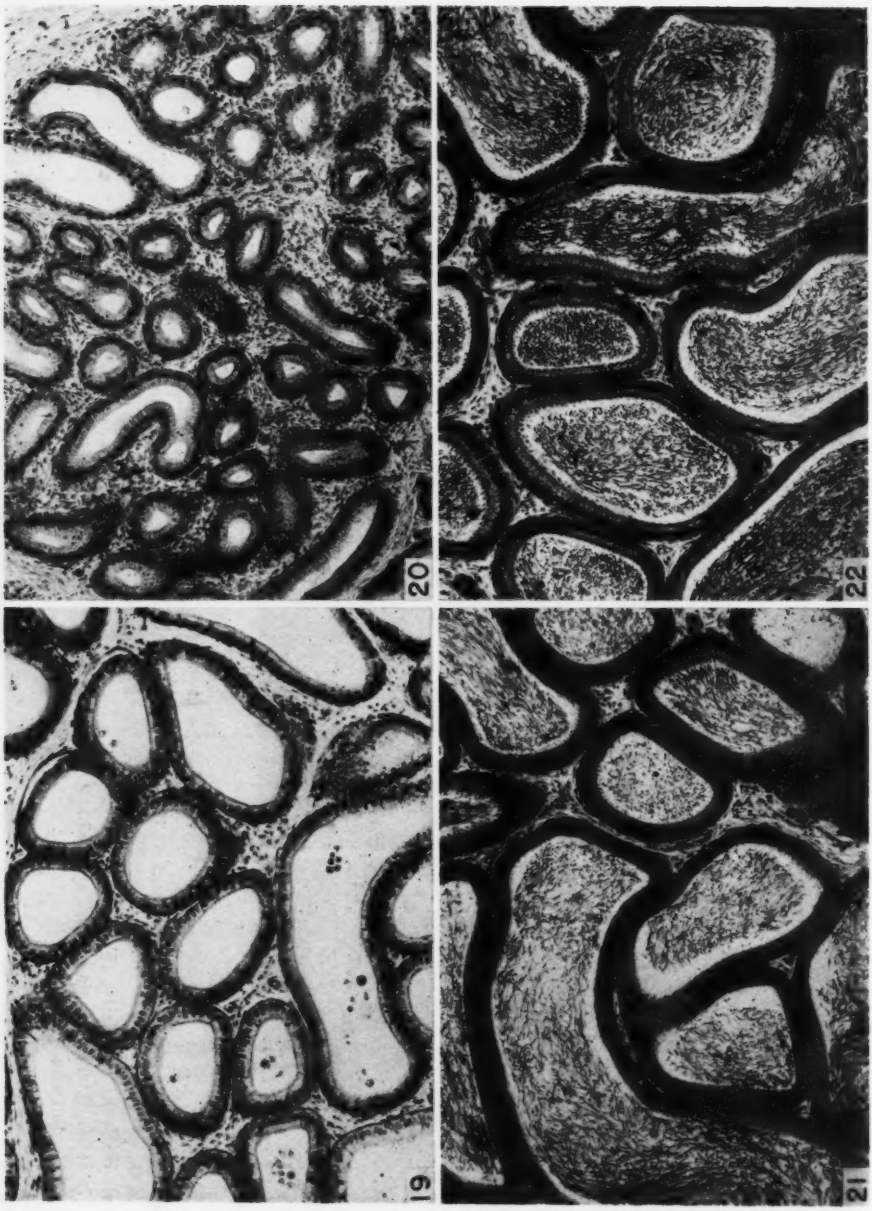

PLATE VII

The preparation of human chorionic gonadotropin (HCG), when given alone, caused spermatozoan formation in all rats in daily doses from 0.010 to 0.050 mg. At doses lower than these, from 0.0025 to 0.005 mg., 40 to 80% of the injected rats produced spermatozoa. The lowest dose tested, 0.001 mg., still caused partial tubular maintenance, a few more spermatocytes remaining adherent than in controls. Partial interstitial cell maintenance resulted when doses of 0.0025 and 0.005 mg. were given; at higher doses, 0.010 mg. and above, the interstitial cells were epithelioid. Accessory organ stimulation was not shown until a dose of 0.010 mg. or more was given.

Due to the marked tubular development resulting from both preparations, it was difficult to show synergistic effects on the tubules from combinations of FSH and HCG, at least at the higher dosages. However, when doses of FSH (0.150 mg. or lower), which alone were marginal for spermatozoan formation were combined with low doses of HCG (0.001-0.0025 mg.) the combination was markedly more effective than either compound alone in enlarging testes, in increasing tubular size and increasing the percentage of rats producing spermatozoa (Figures 16-18).

In regard to the possibility of synergism of the effects of HCG on interstitial cells by combination with FSH, it may be noted that doses of HCG which were sub-minimal for maintenance of interstitial cells (0.001 mg.) or marginal doses (0.0025 mg.) were increased in effectiveness by doses of FSH far below those having any effect on this tissue alone (FSH 0.050 and 0.100 mg.). The effect of HCG on accessory organ development can also be increased by combination with FSH. Doses of HCG showing slight increase in weight of seminal vesicle and prostate (0.010 mg.) can be augmented in their action by doses of FSH in themselves without effect (0.0250 and 0.500 mg.).

It should be noted that the degree of development of the epididymis, as shown by weight and differentiation, paralleled the differentiation of the testicular tubules rather than development of the seminal vesicles and prostate. In groups where low levels of the two hormones gave marked aug-

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DESCRIPTION OF FIGURES

The weight and tubular development of the epididymis follow the testicular tubules rather than the accessory organs. Epididymis, iron hematoxylin, ×88.

FIG. 19. Normal 40 day rat. (Average weight of epididymis 74 mg.)

FIG. 20. Hypophysectomized 40 day rat, 15 days postoperative. (Average weight 34 mg.)

FIG. 21. Normal 55 day rat. (Average weight 182 mg.)

FIG. 22. Hypophysectomized 40 day rat injected subcutaneously with 0.1 mg. FSH +0.0025 mg. HCG daily for 15 days. (Average weight 107 mg.)

mentation of testicular development, the epididymis was also enlarged and contained spermatozoa, whereas the seminal vesicles and prostate remained atrophic as in animals receiving these substances separately (See Table 2 and Figures 19-22).

SUMMARY

Effects of pure pituitary follicle stimulating hormone (FSH) injected subcutaneously, alone and in combination with human chorionic gonadotropin (HCG) have been studied in both male and female hypophysectomized rats.

In *female rats*, FSH caused only follicular development, with no repair of interstitial cells and no corpus luteum formation, even when administered at 10 to 20 times its minimal effective dose.

Development of large follicles and estrous uterus could be induced by low doses of FSH if it was injected simultaneously with HCG. When high levels of FSH were combined with HCG, corpora lutea were formed. These effects were shown before the interstitial cells displayed morphological evidences of repair.

Some augmentation of the effect of HCG on interstitial cells repair was shown by combination with minimal follicle-stimulating doses of FSH.

In hypophysectomized immature *male rats*, FSH caused differentiation of the testicular tubules, with spermatozoan formation. Five or ten times the dose effective on the tubules was necessary for stimulation of interstitial tissue. No effect on weight of accessory organs was obtained at the highest levels tested.

HCG exerted its effects on both tubules and interstitial cells at practically the same low level. A four-fold increase in this dose was sufficient to cause accessory organ weight increase.

Synergistic action of FSH and HCG on the tubules could be demonstrated by simultaneous administration of low doses.

Minimally effective levels of HCG were augmented in their action on interstitial cells and accessory organs by combination with FSH.

The development of the epididymis paralleled the differentiaton of the testcular tubules and occurred in the absence of development of the other accessory reproductive organs.

Note: Since this manuscript was prepared, Van Dyke, P'an and Shedlovsky (*Endocrinology* **46**: 563. 1950) have published an excellent paper describing the preparation of purified FSH from hog pituitaries and its biological properties in both male and female hypophysectomized rats.

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THE ELIMINATION OF A COMMON ERROR IN THE DETERMINATION OF TOTAL 17-KETOSTEROID BY THE m-DINITROBENZENE REACTION*

CHARLES H. SLOAN AND GEORGE H. LOWREY

From the Department of Pediatrics and Communicable Diseases, University of Michigan Medical School

AT THE present time three colorimetric methods for the determination of 17-ketosteroids are in general use. The reaction suggested by Pincus (1943) employs antimony trichloride and acetic acid as color developing agents. The two remaining methods are based on the m-dinitrobenzene reaction suggested by Zimmermann (1935, 1936) and modified by Callow, *et al.* (1938), and Holtorff and Koch (1940). For the determination of total 17-ketosteroid, the Pincus reaction is disadvantageous because dehydroisoandrosterone gives only a faint color with $SbCl_3$ and acetic acid (Pincus, 1943). The Callow modification is impractical for clinical use because of 1) a lesser degree of reproducibility of results (Nathanson, *et al.*, 1943), 2) the necessity of making up fresh alcoholic 2.5 N KOH solution at least once a week (holding the normality in the range from 2.48 to 2.52) as well as keeping reagents and extracts perfectly anhydrous, and 3) the relatively deeply colored blank solution which makes the use of the full galvanometer scale frequently impossible (Wooster, 1943). Friedgood and Berman (1941), Strickler, *et al.* (1941), and Wooster (1943) reported that the calibration for different urine concentrations determined by Holtorff and Koch's method (1940) does not follow Beer's law. Our own observation substantiates their report, and further investigation was undertaken with the purpose of overcoming this disadvantage of an otherwise reliable and convenient procedure.

The purpose of this paper is to present a modification of the Holtorff and Koch procedure. It will be shown that the estimation of 17-ketosteroid by direct comparison of the color developed in samples of urine extract to the standard curve of the pure crystalline hormone necessarily results

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in an underestimation of the total amount of 17-ketosteroid. The method to be presented is designed to correct adequately for this error.

EXPERIMENTAL

The findings in this paper are based upon 113 determinations on 52 urine specimens from 40 male and female individuals of different age groups.

Twenty-four hour urine specimens were collected without preservative. The specimens were stored at 5° C. until determinations were begun. For hydrolysis, extraction, and

TABLE 1. A REPRESENTATIVE SAMPLE OF THE SET-UP OF THE COLORIMETRIC 17-KETOSTEROID DETERMINATION IN DIFFERENT VOLUMES OF URINE EXTRACT UNDER IDENTICAL EXPERIMENTAL CONDITIONS

Tubes No.	Contents of tubes before addition of reagents for color reaction			Color developing reagents added after evaporation		
	Alcoholic urine extract, ml. a.	Alcoholic DHS (250, μG/ml.), ml. b.	Alcohol ml. c.	Alcoholic m-di-nitrobenzene, 1%, ml. d.	Aqueous KOH, 5 N, ml. e.	Alcohol, ml. f.
1–3	.2	—	.6	.4	.2	—
4–6	.2	.2	.4	.4	.2	—
7–9	.4	—	.4	.4	.2	—
10–12	.4	.2	.2	.4	.2	—
13–15	.6	—	.2	.4	.2	—
16–18	.6	.2	—	.4	.2	—
Bu_{1-3}	.6	—	.2	—	.2	.4
S_{1-4}	—	.2	.6	.4	.2	—
$B_{1,2}$	—	—	.8	.4	.2	—
B_T	—	—	.8	—	.2	.4

Explanation:

Tubes numbered from 1 to 3, 7 to 9, and 13 to 15 serve the purpose of determining the L_u values (optical density of urine extract) for three different concentrations of urine extract.

Tubes numbered from 4 to 6, 10 to 12, and 16 to 18 yield the corresponding optical densities of urine extracts with added DHS (L_{Su}).

In the tubes Bu_{1-3}, the optical density of the non-specific color, present before development of the m-dinitrobenzene color, is determined for the highest concentration of urine extract. The values for the lower concentrations are calculated herefrom.

Tubes S_{1-4} contain the standard amount (50 μG) of crystalline DHS, and the color developing reagents, urine extract being absent (L_{Ds}).

Tubes $B_{1,2}$ are reagent blanks used for the zero setting of the colorimeter for the reading of all tubes containing m-dinitrobenzene. Tube B_T is the reagent blank used for the zero setting of the colorimeter for the reading of the tubes Bu_{1-3}.

separation of the 17-ketosteroid and estrogen fraction, the procedure suggested by Salter *et al.* (1948) was followed. The ether extract, containing the 17-ketosteroid fraction, was evaporated to dryness in vacuo, and the residue taken up in a measured amount of absolute alcohol. Different volumes of extract representing multiple equivalents of urine were accurately measured into recalibrated Evelyn colorimeter S-tubes. To one half of each set of tubes representing one urine concentration, .2 ml. of a solution containing 50 μG of crystalline dehydroisoandrosterone (DHS) in absolute alcohol were added. Four additional tubes containing the same amount of crystalline DHS were set up as standards. In order to correct for the color present in the urine extract, but not affected by the color reaction, a series of S-tubes was filled with the volume of urine extract equal to the largest volume present in the tubes to be subjected to the color reaction. By means of addition of appropriate amounts of absolute alcohol, the contents of all S-tubes

TABLE 2. EXPERIMENTAL DATA FOR 12 REPRESENTATIVE 17-KETOSTEROID DETERMINATIONS

Columns U.E. I–III present the experimental data for multiple urine volumes (ratio 1:2:3 respectively). The corrected (c) and uncorrected (u) results are compared. The recovery percentages are calculated from the equation $R = 100u/c$.
The column labeled "Standard" gives the optical density of 50 μG of pure DHS, urine extract being absent.

Test No.	Standard (50μ GDHS)	U.E. I				U.E. II				U.E. III			
		Optical density	17-Keto-steroid, μG	Urine, ml.	Recovery, per cent	Optical density	17-Keto-steroid, μG	Urine, ml.	Recovery, per cent	Optical density	17-Keto-steroid, μG	Urine, ml.	Recovery, per cent
1.	.478	Lu .056 L8u .493 LDu .437	c 6.41 u 5.85	1.0	91.1	Lu .102 L8u .497 LDu .395	c 12.91 u 10.67	2.0	82.6				
2.	.447	Lu .048 L8u .470 LDu .422	c 5.69 u 5.37	.525	94.4	Lu .089 L8u .503 LDu .414	c 10.75 u 9.96	1.05	92.7	Lu .123 L8u .520 LDu .397	c 15.49 u 13.76	1.575	88.8
3.	.465	Lu .206 L8u .668 LDu .462	c 22.29 u 22.15	1.5	99.4	Lu .377 L8u .807 LDu .430	c 43.84 u 40.54	3.0	92.5				
4.	.475	Lu .125 L8u .585 LDu .460	c 13.59 u 13.16	1.5	96.8	Lu .243 L8u .681 LDu .438	c 27.74 u 25.58	3.0	92.2				
5.	.476	Lu .071 L8u .533 LDu .462	c 7.69 u 7.46	1.5	96.9	Lu .133 L8u .568 LDu .435	c 15.29 u 13.97	3.0	91.4				
6.	.457	Lu .129 L8u .373 LDu .444	c 14.53 u 14.11	1.5	97.1	Lu .238 L8u .646 LDu .408	c 29.17 u 26.04	3.0	89.3				
7.	.477	Lu .152 L8u .607 LDu .455	c 16.70 u 15.93	1.5	95.4	Lu .294 L8u .735 LDu .441	c 33.33 u 30.82	3.0	92.5				
8.	.497					Lu .156 L8u .611 LDu .455	c 17.14 u 15.69	2.0	91.5	Lu .227 L8u .670 LDu .443	c 25.62 u 22.84	3.0	89.1
9.	.484	Lu .081 L8u .554 LDu .473	c 8.56 u 8.37	1.0	97.8	Lu .151 L8u .592 LDu .441	c 17.12 u 15.60	2.0	91.1	Lu .215 L8u .654 LDu .439	c 24.47 u 22.21	3.0	90.8
10.	.481	Lu .069 L8u .547 LDu .478	c 7.22 u 7.17	1.5	99.3	Lu .123 L8u .556 LDu .433	c 14.20 u 12.79	3.0	90.1				
11.	.459	Lu .080 L8u .528 LDu .448	c 8.93 u 8.71	3.0	97.5	Lu .146 L8u .568 LDu .422	c 17.30 u 15.90	6.0	91.9				
12.	.473	Lu .047 L8u .514 LDu .467	c 5.03 u 4.97	.96	98.8	Lu .084 L8u .535 LDu .451	c 9.31 u 8.88	1.92	95.4	Lu .123 L8u .540 LDu .417	c 14.75 u 13.00	2.88	88.0

were adjusted to equal volumes, and blank tubes were made up containing the same amount of alcohol (Table 1, columns a–c).

All tubes were evaporated to dryness in vacuo, and the residues were taken up in .4 ml. of a 1 per cent solution of m-dinitrobenzene in absolute alcohol, with the exception of those tubes which were to serve the purpose of correcting for the colored substances in the urine extract. Into the latter, and one blank tube, were measured .4 ml. of absolute alcohol instead of the m-dinitrobenzene solution (tubes B_u and B_T in Table I). After addition of .2 ml. of 5 N aqueous KOH to all tubes, and shaking, the entire series was incubated in the dark at 25° C. for 60 minutes. The incubation time of 60 minutes was selected since it was observed that a longer incubation period does not result in an appreciable increase of color development. After incubation, 10 ml. of absolute alcohol were added to each tube, the contents well mixed by shaking, and the developed color was read within 15 minutes in an Evelyn photocolorimeter, using a Rubicon filter having a maximum transmission at about 520 mμ.

RESULTS

In Table 2 are listed representative results obtained by means of the described method. The optical densities indicated in this table represent the samples containing urine extract (L_u), and urine extract plus the standard amount (50 μG) of crystalline DHS (L_{Su}), respectively. L_{Du} values are found by subtracting L_u from L_{Su}, and represent the increase in color development exclusively due to the addition of the known amount of DHS to the specific sample. The "Standard" values are based on the color developed by 50 μG of DHS in the pure reagent mixture, urine extract being absent. Most of the figures represent average values for determinations done simultaneously in triplicate, a few were run in duplicate or quadruplicate. From the data presented it is evident that the L_{Du} values determined for different concentrations of the same urine sample decrease with increasing urine concentration. It is obvious that the addition of equal amounts of crystalline 17-ketosteroid, under otherwise exactly identical conditions, yields less color in the presence of an increasing urine concentration, while the reaction mixture containing the pure DHS, urine extract being absent, yields the highest optical density (Table 2, column labeled "Standard"). The results shown under "17-Ketosteroid, μG" give the corresponding amounts of 17-ketosteroid, expressed in micrograms of DHS. The "uncorrected" results (u) are calculated using the colorimeter readings of the pure DHS samples (L_{Ds}) for comparison. For the calculation of the "corrected" values (c) the color developed by the DHS added to the urine extracts (L_{Du}) were employed. The 17-ketosteroid concentration (C) is calculated from the equations:

$$C_{corrected} = \frac{L_u \cdot D}{L_{Du}} \qquad C_{uncorrected} = \frac{L_u \cdot D}{L_{Ds}}$$

(D = μG of DHS added to the urine extract, or used as pure standard,

respectively). The uncorrected results for 17-ketosteroid show a steadily increasing apparent loss for increasing urine concentrations. The corrected values follow the expected ratio of the concentrations of the samples.[1]

On the basis of the results reported above, it was assumed that the deviation from Beer's Law which is observed for different urine concentrations in the determination of 17-ketosteroid by Holtorff and Koch's method was due to different degrees of depression of color development for the concentrations in question. In order to validate this assumption, different amounts of crystalline DHS were added to identical samples of urine extract, and it was observed that the standard curves thus obtained showed linearity according to Beer's Law. However, the linear standard curves obtained in the described manner for different concentrations of the same urine specimen were not parallels, but with increasing urine equivalent the angles of their slope decreased (Figure 1). These findings clearly indicate that the amount of color developed by the 17-ketosteroids in the Holtorff and Koch modification of the Zimmermann method is greatly influenced by urinary substances other than 17-ketosteroid present in the final test solution. Direct comparison of the color developed in urine extracts to the color developed in samples of crystalline standard therefore results in an underestimation of hormone present. This is because the presence of urine extract, as well as its concentration, constitutes important variable factors altering the conditions of color development. For this reason, it is indicated to run the known standard not separately, but directly in the appropriate concentration of urine extract, therewith obtaining a standard value representing the color development of a known amount of 17-ketosteroid under the experimental conditions. Without requiring additional work or equipment, this can easily be done in the manner described above, i.e., by addition of a known amount of crystalline 17-ketosteroid to the exact volume of urine extract to be assayed. Subtraction of the optical density of the urine extract (L_u) from the sample containing the additional amount of crystalline 17-ketosteroid (L_{su}) will give directly the optical density value of the color yielded by the added

[1] Statistical analysis of the first 36 consecutive determinations showed the following results:

	Ratio 2.00 (N = 34)		Ratio 1.5 (N = 14)	
	corrected	uncorrected	corrected	uncorrected
$\bar{X}$	1.99	1.89	1.49	1.43
t	.50	8.39	.75	4.34
P	> .50	< .001	> .10	< .001

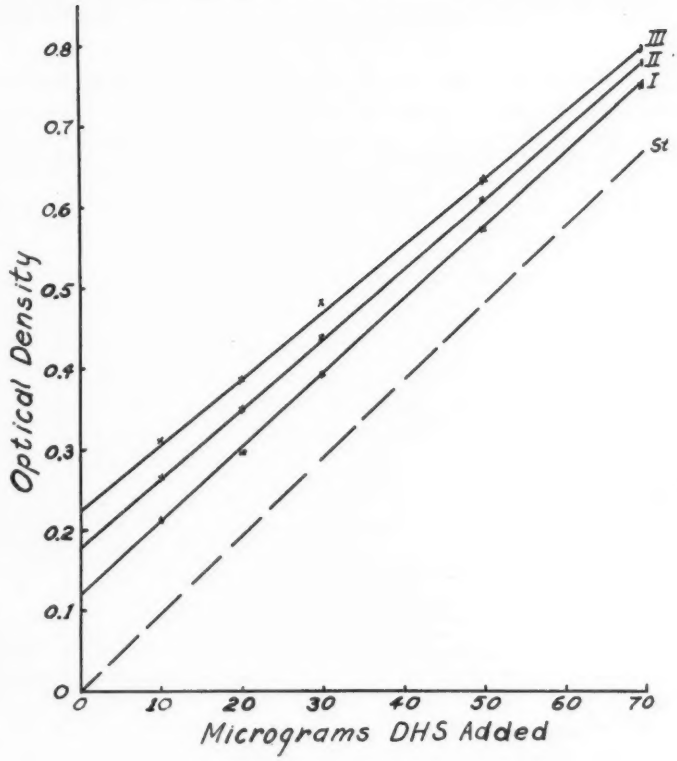

FIG. 1

I: 2 ml. of urine
II: 3 ml. of urine
III: 4 ml. of urine
St: pure DHS, urine being absent.

amount of crystalline steroid (L_{Du}) in the given concentration of that specific urine extract.

Although, at the present time, we have no clear knowledge of the mechanism of this color inhibiting action and its agents, it may in future investigation become of increasing significance because of the possibility that other hormone compounds or their degradation products cause the observed inhibition phenomena. In this connection, it is interesting to note that a number of estrogen determinations by means of the Kober reaction, employing a procedure essentially similar to the one outlined above for the 17-ketosteroid determination, also showed an increasing inhibition of

color development with increasing urine concentration (study in progress).

Recovery experiments and determinations made in order to establish normal 17-ketosteroid ranges for different age and sex groups (unpublished data, work in progress) seem to indicate that, on application of the modification suggested above, the overall recovery is significantly higher than the recovery obtained by means of the conventional Holtorff and Koch method.

SUMMARY

A procedure for the determination of total 17-ketosteroid by means of the m-dinitrobenzene method is presented. It is shown that the conventional estimation of 17-ketosteroid by direct comparison of the color developed in samples of urine extract to the standard curve of pure crystalline hormone results in an underestimation of the total amount of 17-ketosteroid. The described method corrects for this error.

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THE RELATION OF THE ADRENAL TO RED BLOOD CELL FRAGILITY[1]

HERBERT MEGEL AND ALBERT S. GORDON[2]

From the Department of Biology, Washington Square College of Arts and Science, New York University, New York City

ALTHOUGH the evidence is clear that the endocrine system regulates certain phases of blood formation (reviewed by Gordon and Charipper, 1947), its significance in blood destruction processes still remains to be elucidated. Of the organs examined, the spleen has been the subject of most investigation and in this connection, Gordon and Kleinberg (1937) and Gordon, Kleinberg and Ponder (1937) have reported a decrease in red cell fragility following splenectomy both under *in vitro* and *in vivo* conditions. That the adrenal may also be implicated in blood destruction mechanisms has been indicated in reports by Reid (1932) and more recently by Gordon and Piliero (1950) who have detected an increased resistance of the red cell of the adrenalectomized animal towards hypotonic saline.

It was felt that a more extensive investigation of the relation of the adrenal to red blood cell fragility might provide a clue as to the general significance of the endocrine system in blood destruction processes. The present work is concerned therefore with a study of the factors involved in the demonstrated increased resistance of the red blood cell to hypotonic saline following adrenal ablation.

MATERIALS AND METHODS

Female rats of a modified Long-Evans strain, weighing 140–160 grams, were adrenalectomized and employed 2 weeks after the operation, at which time the red cell fragility alterations are seen to good advantage (Gordon and Piliero, 1950). Blood was withdrawn by cardiac puncture and heparinized. Both experimental and control animals were kept on a standard laboratory ration and all adrenalectomized animals received 1% NaCl solution for drinking water. The hemolysis techniques used were essentially those of Ponder (1948). The procedures employed for the 5 series of experiments conducted were as follows.

Series I. Studies with washed red blood cells

The blood was centrifuged for 15 minutes and the plasma decanted. The cells were washed 3 times with 1% NaCl, centrifugation being carried out after each washing. A 2.5% red blood cell suspension in 1% saline was then made from the packed erythro-

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cytes. The lytic system was so prepared that the addition of 0.4 cc. of the red cell suspension to 1.6 cc. of the previously adjusted hypotonic saline solution would bring the total volume of 2.0 cc. to the desired tonicity. These hypotonic saline suspensions were incubated for 30 minutes in a water bath thermostatically regulated at 30 degrees C., after which time the tubes were centrifuged, the supernatant fluids removed, and the hemoglobin concentrations read in a Klett photoelectric colorimeter. The 100% lysis control tube was prepared by adding 0.4 cc. of the blood suspension to 1.6 cc. of water. The extent of hemolysis corresponding to the hypotonic dilutions was expressed as a percentage of the control tube. In these experiments the washed cells from 8 control and 7 adrenalectomized animals were employed.

Series II. Experiments with whole blood

The method employed in this Series was essentially that used in Series I except that a drop of whole blood equivalent, in red cell numbers, to 0.4 cc. of the 2.5% blood suspension was introduced into 2.0 cc. of the hypotonic saline. The drop of blood, calibrated as equivalent to 0.018 cc. was delivered from a marked capillary tube, the same tube being employed throughout the experiment. The bloods of 7 normal and 6 adrenalectomized rats were used in this series. Also studied were 4 adrenalectomized animals injected with 0.5 cc. of lipoadrenal extract (Upjohn—1 cc. equals 40 rat units) and 4 adrenalectomized rats injected with 3 mg. of Compound E (17-OH-11 dehydrocorticosterone acetate)[3] daily for 2 weeks.

Series III. Erythrocyte and plasma exchange studies

This series involved obtaining per cent hemolysis data using the same procedure as outlined for Series I except that the medium for the 2.5% red cell suspension consisted of 1 part plasma to 3 of saline instead of entirely saline. Each hemolytic system of 2.0 cc. contained therefore 0.1 cc. plasma. Four cell suspensions were prepared: 1) normal cells in normal rat plasma, 2) normal cells in adrenalectomized rat plasma, 3) adrenalectomized animal cells in normal plasma and 4) adrenalectomized animal cells in adrenalectomized rat plasma. These suspensions were incubated at 30 degrees C. for 15 minutes. Four tenths of a cc. of these blood suspensions was added to 1.6 cc. of the different hypotonic solutions equivalent to those used in Series I and II. The hemolytic systems were incubated for 30 minutes at 30 degrees C., centrifuged and the supernatant fluids read in the colorimeter against a control for 100% lysis. Six normal and six adrenalectomized rats provided the erythrocytes and plasmas for this Series.

Series IV. Factor adsorption

During the course of the experiments, it became evident that the plasma contained a factor which inhibited hypotonic hemolysis and furthermore, that this factor appeared to act by being adsorbed upon the red cell. The following method was employed to amplify this point. A total of 4 adrenalectomized and 8 control rats was used. For each of the 4 runs made, blood was withdrawn from one adrenalectomized and 2 control animals. The plasma was separated from the packed cells after centrifugation and the normal red cells were washed 3 times with isotonic saline. One sample of normal red cells was diluted back to its original volume with isotonic saline. Six parts of isotonic saline were added to 1 part of plasma from the adrenalectomized animal and then 0.1 cc., 0.2 cc., 0.4 cc. and 0.8 cc. of packed normal cells were added to 4 tubes, each containing 3.0 cc.

[3] We wish to thank Drs. H. J. Robinson and A. Gibson, Merck and Co., for the supplies of Compound E furnished.

of the saline-diluted adrenalectomized rat plasma. These suspensions were incubated for 15 minutes and centrifuged. The diluted plasmas were now separated from the added normal red cells and 0.9 cc. of each of these added to its respective test tube. A drop, equivalent to 0.018 cc., of normal blood which had been diluted back to its original volume with saline was added to each tube and incubated again for 15 minutes. After this time 1.1 cc. of water was introduced into each tube resulting in a 0.45% hypotonic solution, each tube now containing 0.129 cc. of adrenalectomized rat plasma which had been in contact with the normal cells. These suspensions were incubated at 30 degrees C. for 30 minutes following which time they were centrifuged and the supernatants read in the colorimeter. These values were then compared with the 100% lysis tube. Plasma to which no cells were added as well as 0.45% NaCl also served as controls.

Series V. Red cell volume changes

This series was run to gain information concerning the osmotic properties of the red cells of the adrenalectomized animals. The procedures used were essentially those employed by Guest and Wing (1939, 1946) in which heparinized blood is drawn to the 0.03 cc. mark in a large Van Allen hematocrit tube and diluted fully (about 8.0 cc.) with the appropriate salt solution. After a 30 minute incubation period the tubes were centrifuged for 15 minutes at 2500 RPM. The packed cell volumes were read directly from the stem of the tube and the per cent hemolysis values were obtained from the supernatants in the colorimeter. Where partial lysis had occurred, the observed volume was corrected for the loss of cells by lysis by the following equation:

$$\frac{\text{Volume of Packed Cells}}{\text{Per cent of Non-Hemolyzed Cells}} \times 100 = \text{Corrected Volume}$$

Values for per cent volume changes were obtained by dividing the volume of packed cells (experimental) by the volume of packed cells (in 1% saline) and multiplying by 100. A total of 6 normal and 6 adrenalectomized animals provided the material for this series.

RESULTS

Series I, II and III

It was considered advisable in describing the results to place the major emphasis upon one critical tonicity, namely 0.45% NaCl or its equivalent, since the maximum effects are best seen at this dilution. The data in Fig. 1 indicate that the resistance of the washed cells of adrenalectomized rats is not significantly different from that of control cells when tested against hypotonic saline. However, the results for the whole blood series make 2 points clear: 1) red blood cells in their own plasma are more resistant than washed cells, and 2) the red cells of adrenalectomized rats when added as whole blood, are more resistant to hypotonic lysis than are normal cells. From Table 1, it may also be seen that treatment of adrenalectomized animals with lipoadrenal extract or cortisone restores red cell fragility to normal values.

Observation of Fig. 1 will show that the incubation of normal red cells in the plasma of adrenalectomized rats increases their resistance towards

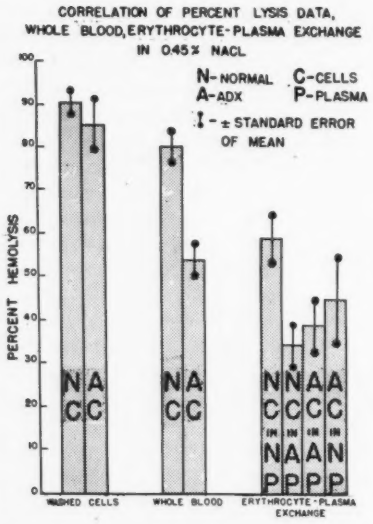

FIG. 1. Histograms indicate per cent hemolysis values in 0.45% NaCl for washed cells, whole blood, and washed cells suspended in different plasmas.

hypotonic saline and to a greater extent than if the cells are suspended in the plasma of normal rats. Similarly, there is a tendency for the cells of adrenalectomized rats to be less resistant in normal plasma than when they are suspended in their own plasma. Although this latter result, upon analysis, does not prove to be statistically significant, in no case does the decreased resistance noted here approach that displayed by *normal* cells in normal plasma. These indications that the factor responsible for the increased resistance to hypotonic saline is present in plasma and that this

TABLE 1. RESISTANCE OF WHOLE BLOOD TO HYPOTONIC SALINE.
PER CENT HEMOLYSIS (MEANS ± STANDARD ERRORS)

NaCl tonicity	Normal (7)[1]	Adx[2] (6)[1]	Adx + lipo[3] (4)[1]	Adx + cort[4] (4)[1]
0.000	100 ± 0.0	100 ± 0.0	100 ± 0.0	100 ± 0.0
0.400	96 ± 0.6	91 ± 1.3	98 ± 0.2	96 ± 2.1
0.425	95 ± 1.9	78 ± 4.2	93 ± 2.4	92 ± 2.9
0.450	77 ± 1.9	53 ± 3.2	78 ± 4.1	82 ± 4.4
0.475	45 ± 7.7	34 ± 7.3	41 ± 3.9	57 ± 9.3
0.500	24 ± 2.1	16 ± 4.1	9 ± 1.3	34 ± 12.1
0.550	3 ± 0.7	3 ± 0.7		
0.600	1.4 ± 0.6	2.6 ± 0.4		
1.000	1.1 ± 0.4	1.5 ± 0.3		

[1] Number of animals.
[2] Bilateral adrenalectomy.
[3] Adrenalectomy + Lipoadrenal Cortical Extract.
[4] Adrenalectomy + Cortisone.

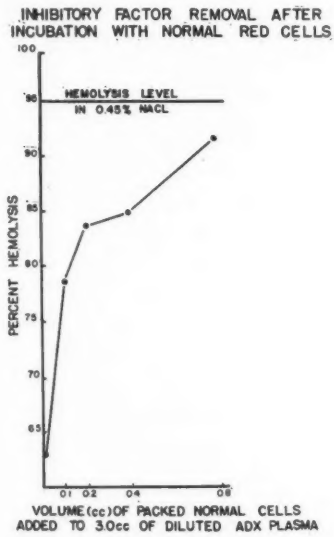

FIG. 2. Curve represents inhibitory effects of diluted plasma on hypotonic lysis after incubation with different numbers of normal washed red cells.

factor operated by becoming "adsorbed" to the cell stimulated the next series of experiments.

Series IV

Incubation of washed normal red cells with diluted plasma from adrenalectomized animals results in the disappearance of the factor inhibitory to hypotonic lysis. Although the quantity of factor removed increases as greater numbers of red cells are added (Fig. 2), strict adherence of the data to the adsorption isotherm does not occur.

Series V

Although the differences are not significant, there appears to be a consistent tendency for the cells of the adrenalectomized rats in the 3 hypo-

TABLE 2. RED BLOOD CELL VOLUME CHANGES IN HYPOTONIC SALINE. PER CENT VOLUME INCREASES (MEANS ± STANDARD ERRORS)

NaCl tonicity	Normal (6)[1]	Adx[2] (6)[1]
0.50	154 ± 12.5	146 ± 5.1
0.55	148 ± 3.6	142 ± 4.2
0.60	138 ± 2.8	135 ± 1.6
1.00	100 ± 0.0	100 ± 0.0

[1] Number of animals.
[2] Bilateral adrenalectomy.

tonic dilutions studied (Table 2), to swell to a lesser extent than normal cells.

DISCUSSION

The experiments described here confirm the observations of Gordon and Piliero (1950) and Piliero, Landau and Gordon (1950) who have reported that the blood of adrenalectomized rats is more resistant to hypotonic lysis than that of normal animals. In addition, the results indicate that the factor (or factors) responsible for this increased resistance exists in the plasma, and to a greater extent in that of the adrenalectomized than in the normal animal. Moreover, this factor appears to act by becoming adsorbed to (and/or absorbed by) the red cell. Experiments performed in this work which support this conclusion include the following: 1) washed cells do not display these differences in fragility, 2) the resistance of washed cells is increased when they are immersed in plasma and to a greater degree in that of adrenalectomized than of normal animals, 3) incubation of red cells in the plasma of adrenalectomized rats is accompanied by a disappearance of the inhibitory factor from the plasma. Addition of greater numbers of cells, with a consequent increase in surface exposed, results in a removal of greater quantities of the inhibitor. Similarly a 4th experiment performed recently and not included in the Results, indicates that preliminary incubation of washed red cells with plasma, under isotonic conditions, increases their resistance to subsequent lysis to a much greater extent than if the red cells are added directly to already prepared hypotonic solutions containing identical amounts of plasma.

The nature and mechanism of action of this inhibitory phenomenon can only be conjectured upon at the present. Brinkman and van Dam (1920) reported that normal red blood cells are more resistant when washed in Brinkman's physiological saline than unwashed cells. These authors postulated that the solution washed off a lysis accelerating factor (lecith n), leaving behind in the plasma an inhibitory factor (cholesterol). Saslow (1932) was unable to confirm this work and Gordon and Kleinberg (1937) have observed that high concentrations of cholesterol, added directly. are without effect on erythrocyte resistance to hypotonic lysis. The possibility exists, however, that the inhibitory capacity of plasma is due to a multiplicity of factors, of which cholesterol may participate as one. Thus alteration in the type and concentration of the protein present in plasma may also be a contributory factor to this phenomenon.[4] Whatever the nature of the inhibitory complex may be, the present experiments establish that

[4] Protein factors in serum and plasma, inhibitory to saponin and sodium taurocholate lysis, have been described by Ponder (1923, 1925) and Ponder and Gordon (1934).

it appears in greater concentrations in the adrenalectomized animal and is prevented from accumulating in excess by the administration of adrenal cortical factors.

It is of interest to speculate upon the possible mechanisms of action of the plasma inhibitory factor. One possibility is that it induces an alteration in the shape-volume relationship of the cell. Assuming no change in membrane elasticity, a more hypotonic solution would be required to lyse a flat cell than one with a normal shape. Such a cell would swell to a greater percentage above its initial volume before the critical volume preceeding lysis is reached. A change of precisely this type has been observed for the guinea pig red cell following splenectomy (Gordon, Kleinberg and Ponder, 1937). This explanation, however, is not in accord with the results of the present experiments since here a tendency towards a *lesser* degree of swelling in hypotonic media has been observed for the red cells of the adrenalectomized rat.

Another mechanism concerns the possibility that the plasma factor induces an increase in permeability of the erythrocyte membrane resulting in a net loss of osmotically active particles from the cell with a consequent increased resistance to hypotonic saline. In this connection, an increase in the permeability of the red cell to cations following adrenal removal has actually been reported (Kerr, 1929). However, more proof for the validity of this concept is required before it can be accepted.

A third possible mechanism may operate by decreasing the permeability of the red cell membrane to water. The only evidence for an agent affecting the selective permeability of the plasma membrane has been presented by LeFever (1946) and by Jacobs (1950) who have demonstrated an inhibitory action of the copper ion upon the penetration of glycerol through the membrane. No experiments have as yet identified a substance which will affect the permeability of the membrane specifically to water and so this hypothesis remains also to be proved.

As a final point, it should be mentioned that Laser (1949) has reported the isolation of a fatty acid from plasma, blood cells, liver and other tissues capable of producing lysis in concentrations as low as 1 in 600,000. Experiments should be performed to determine the concentration of this factor in the plasma and blood cells following adrenalectomy because of its possible significance in the anemia and increased red cell resistance observed in the adrenalectomized animal.

SUMMARY

The resistance of the blood to hypotonic lysis is increased following adrenal removal. This effect is not present when washed cells instead of whole blood is employed.

The plasma is demonstrated to contain a factor(s) which inhibits hypotonic lysis. It is present to a greater extent in the plasma of the adrenalectomized than in that of normal rats. Evidence is presented to show that the plasma factor operates through an adsorption (and/or absorption) mechanism.

The possible nature and mechanisms of action of the factor inhibitory to hypotonic lysis are discussed.

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POTASSIUM DEFICIENCY AS CAUSE OF THE SO-CALLED RHEUMATIC HEART LESIONS OF THE ADAPTATION SYNDROME*

ERNST PESCHEL, M.D., BERNARD BLACK-SCHAFFER, M.D., AND CLOTILDE SCHLAYER, PH.D.

From the Departments of Medicine and Pathology, Duke University, School of Medicine, Durham, North Carolina

IN THE course of experiments designed to study the relationship of diet and hypertension, various techniques of inducing hypertension in rats were used including Selye's method which he describes in his communication on pathogenetical correlations between periarteritis nodosa, renal hypertension and rheumatic lesions (Selye and Pentz, 1943). This method consists of overdosage with desoxycorticosterone acetate (DCA) with or without high sodium chloride intake in unilaterally nephrectomized rats. Selye found that severe overdosage with DCA reproduces in the rat morphological lesions similar to those seen in periarteritis nodosa, malignant hypertension and rheumatic fever. He concludes that these diseases are, at least partly caused by an abnormal adaptive response of the adrenal cortex and represent diseases of adaptation.

In the first series of our experiments when rats with an average weight of 168 grams were treated with DCA overdosage following unilateral nephrectomy, hypertension was only rarely produced, and periarteritis nodosa was not seen. (In further experiments using younger rats of 80 to 124 grams as in Selye's original work, hypertension did consistently develop and periarteritis nodosa was occasionally found; cardiac lesions occurred only in some animals, corresponding with the observations reported in this paper.)

Despite the failure to produce hypertension in the earlier experiment, cardiac lesions were noted which were identical with those reported by Selye and Pentz (1943) as resembling rheumatic heart disease in man. However, the experimental set-up and our past experience (Smith, Black-Schaffer and Lasater, 1950) enabled us to show that these lesions are not related to rheumatic fever, etiologically or pathogenetically, but are the consequence of potassium deficiency.

METHODS

Female albinos of the Osborne-Mendel strain weighing between 112 and 219 grams, average 168 grams, were used. As indicated in Table 1, 39 rats were divided into eight groups of 4 to 5 animals each. The weight distribution among the groups was approximately equal.

Each group was placed on a diet consisting of one well defined food stuff (such as beef, fat cheese, rice, potatoes, etc.) supplemented by vitamins. The vitamin supple-

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ments per rat and per day were: thiamine 40 γ; riboflavin 100 γ; niacin 500 γ; pyridoxine 40 γ; calcium pantothenate 150 γ; choline 7 mg.; ascorbic acid 2.5 mg.; β-carotene 200 γ; vitamin D 5 USP units; α-tocopherol 500 γ. Control animals, not treated with nephrectomy or DCA and placed on rice without additional sodium chloride, were killed after 25, 50, 100, 200, 400 and 600 days respectively. The microscopic examinations did not reveal pathological changes in any organ. This indicated that the vitamin supplements were sufficient.

The daily food intake of the individual animal was measured by comparing the weight of the food container when filled and ready to be put into the cage, and when taken out after 24 hours. Detectable spilling of food did not occur. Raised wire mesh bottoms in the

TABLE 1. EFFECT OF DIETS WITH DIFFERENT POTASSIUM CONTENT ON SURVIVAL TIME OF DCA-TREATED, UNILATERALLY NEPHRECTOMIZED RATS

Diet		Fat cheese	Rice	Bread	Eggs	Beef	Dog chow	Peas	Potatoes
Mg. potassium in 24 hr. diet		7	12	22	31	59	160	167	188
Survival time (days)	Tap water	35	**	45	*	**	*	*	*
		40	**	*	*	**	*	*	*
	1% NaCl solution	14	19	30	A.	33	*	*	*
		18	20	31	33	44	*	*	*
		24	21	A.	A.	**		*	*

* Killed after 75 days. ** Killed after 425 days. A. Died accidentally

cages prevented access to excreta. Average food intakes for each diet group were calculated from the food consumptions of the individual rats.

Tap water was the drinking fluid for two animals in each group while the remainder received a 1% sodium chloride tap water solution.

DCA (in aqueous suspension, 20 and 30 mg. per cc. respectively) was injected twice daily for 40 days as follows: 8.0 mg. per diem, subcutaneously, for the first 10 days and 12.0 mg. per diem, for the following 30 days. The injections were started ten days after the special diets were begun. The diets with or without saline were continued without change after the DCA was discontinued. All survival times were calculated from the beginning of DCA administration.

RESULTS

Only one animal developed persistent hypertension which varied between 150 and 178 mm. Hg (systolic). This animal was on beef diet with additional high sodium chloride intake. Occasional blood pressure readings of 138 to 168 mm. Hg were obtained in four others—one from the bread group, two from the potato group, and another one from the beef group, all on additional high sodium chloride intake.

Fourteen animals died between the 14th and the 46th day. Three additional rats died accidentally: one was suffocated on the seventh day, the two others died during cardiac puncture on the 45th day. Seventeen of the 22 survivors were killed on the 75th and the remaining 5 on the 425th day after the start of the experiment.

All the rats who died before the 46th experimental day (with the exception of 2 of the 3 who died accidentally) developed to a greater or lesser degree some or all of the following symptoms suggesting cardiac failure: dyspnea, cyanosis, peripheral edema, ascites, pleural effusions. There was no indication of infections of the respiratory system, but the microscopic examination showed passive congestion of lungs, liver and spleen in all the 14 rats who died spontaneously, and in one who died accidentally on the 45th day.

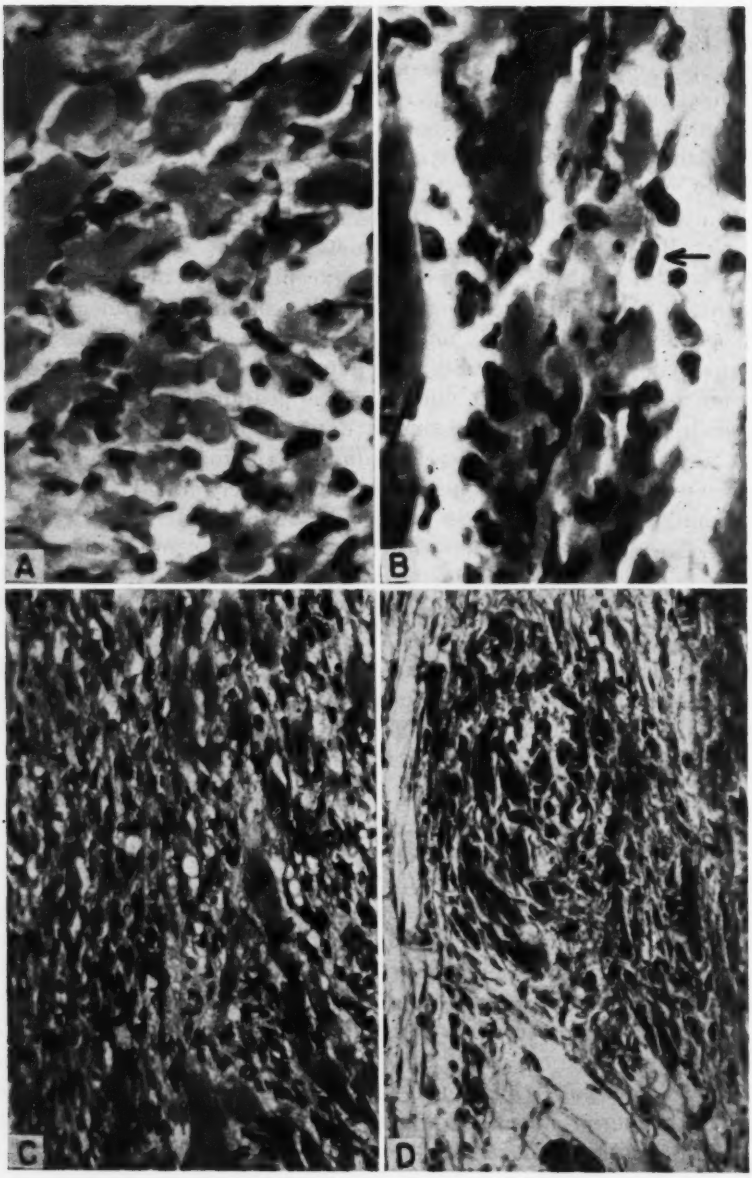
The myocardium of several of these animals showed greyish foci measuring up to about 3 mm. in greatest diameter.

The hearts of all the rats (with the exception of 2 of the 3 who died accidentally) who died before the 46th day of the experiment showed, histologically, a qualitatively uniform lesion—myocardial necrosis and an accompanying cellular reaction. The development of these lesions could be studied by comparing the microscopic appearance in different animals representing different stages of cardiac damage. The process is first visible in the fibers subjacent to the endocardium of the four chambers. The left ventricular wall is most frequently and extensively involved followed in diminishing order by the right ventricular myocardium and that of the auricles. The myocyte striations lose their distinctness and the sarcoplasm assumes a homogenous hyaline appearance (Figure 1A). The changed sarcoplasm then breaks up into smaller and larger eosinophilic particles with ultimate fragmentation of the entire myocyte (Figure 1B). The nucleus meanwhile becomes pyknotic or swells and disappears. Individual fibers separated by seemingly normal myocytes undergo this change particularly in the inner third of the left ventricular myocardium. The Masson trichrome stain (Mallory, 1938) is very helpful in demonstrating the eclectic nature of the necrosis since in its earliest manifestations there is no cellular exudate or connective tissue reaction to focus attention. Nowhere may damaged vessels or thrombosis be seen.

The fibrocytes and capillary endothelial cells respond to the necrosis by proliferation (Figure 1B). Among the fibroblasts are many characteristic Anitschkow cells, a small number of polymorphonuclear leucocytes and some monocytes. These last manifest their phagocytic property and enlarge to typical macrophages.

As the process advances large areas in the inner third of the left ventricle, and to a lesser extent elsewhere, show complete replacement by fibroblasts and Anitschkow cells (Figure 1C). Multiplication of these elements is rapid, and numerous mitotic figures are readily found.

The oldest lesions, usually in the papillary muscles and the trabeculae carneae show a very slight increase in reticulin fibers: an early indication of eventual scarring.


A
B
C
D

In no instance was a valvulitis seen.

No trace of any myocardial lesion resembling that described above was found in the hearts of the rats killed after 75 days and 425 days respectively.

To one acquainted with the lesions of severe hypopotassemia in the rat it is at once apparent that the cardiac changes produced in this experiment are identical with those easily produced in the same species by means of a low potassium diet (Schrader, Prickett and Salmon, 1937; Thomas, Mylon and Winternitz, 1940; Follis, Orent-Keiles and McCollum, 1942; Kornberg and Endicott, 1946). Furthermore, it is just as apparent that both, in all respects, are similar to those depicted and described by Selye and Pentz (1943) as analogues of rheumatic myocarditis in man.

To test the conclusions arrived at from a consideration of the pathologic anatomy of the hearts, the potassium contents of the various diets were estimated according to the tables of McCance and Widdowson (1947). Table 1 lists the average amount of potassium actually consumed for each category. Correlating survival time and number of survivors with the average daily consumption of potassium reveals that rats receiving diets with low amounts of potassium died in larger numbers and sooner than those on a higher potassium intake.

The serum potassium level of 10 normal control rats, determined gravimetrically, was found to be 5.17 ± 0.29 m.eq./l. (average, with standard deviation). On the 75th day, i.e. 35 days after DCA was stopped, four of the tap water survivors—2 from the egg group, 1 from the pea group and the fourth from the potato group (all relatively high potassium diets)—showed serum potassium levels of 4.30, 4.71, 3.84 and 4.43, average 4.32 ± 0.36 m. eq./l. In contrast 3 rats with high sodium intake (saline drinking water and potato diet) had serum potassium concentrations of 3.94, 2.56 and 3.99, average 3.50 ± 0.81 m. eq/l. Statistical analysis shows both these averages to be significant decreases from the normal, P being less than 0.01. It is apparent therefore that continuing the high sodium chloride intake even after the DCA has been discontinued maintains the decreased

FIG. 1. A. The upper half illustrates early necrosis of the myocytes indicated by loss of striations, homogenization of the sarcoplasm and disappearance of the nuclei. The lower portion shows the earliest manifest connective tissue cell and monocyte response to the myocardial necrosis. B. Progressive necrosis of the myocardium is indicated by complete fragmentation of myocytes and the further proliferation of fixed tissue cells, many of them Anitschkow cells (arrow), and monocytes. C. The disappearance of dead myocardium is apparent as well as its replacement by rapidly proliferating fibroblasts and Anitschkow cells. D. In contrast to the above, photograph D presents an Aschoff nodule in the heart of a child. Note the interstitial location, the relatively well defined appearance of the nodule and the characteristic fibrinoid change of the connective tissue; all absent in A, B and C above.

potassium concentration in the serum. However, on these diets, the hypopotassemia caused by these factors alone is not severe enough to produce death of the animals or myocardial lesions.

As an additional control, we placed twelve rats each weighing between 86 and 112 grams (average 98 grams) on a diet identical to that of group 2 (rice). All twelve received as drinking water a 1% sodium chloride solution. To this, for six of the animals, potassium chloride to make up a 1.27% solution was added. Desoxycorticosterone acetate (in aqueous suspension, concentration 15 mg. per cc., dose 6.0 mg. per day) was injected twice daily subcutaneously into all twelve rats.

Of the six animals without potassium chloride supplement, four died spontaneously, three on the 23rd day after the start of the experiment, the fourth on the 27th day. The remaining two were killed on the 28th day. Two of the rats dying on the 23rd day did not show any recognizable cardiac lesions, the other four revealed characteristic lesions as described above.

All six rats receiving potassium chloride supplement were killed on the 28th experimental day; none showed cardiac necrosis.

The serum potassium concentrations of three of these control rats without potassium chloride supplement were found at the time of death to be 1.71, 2.81 and 2.16, average 2.23 ± 0.55 m. eq./l. The serum potassium concentrations of four rats with potassium chloride supplement were 4.13, 4.51, 3.20 and 5.12, average 4.24 ± 0.80 m.eq./l. Both these averages represent statistically significant decreases from the normal. Ten normal rats showed an average serum potassium level of 5.17 ± 0.29 m.eq./l.

DISCUSSION

It has been found that the addition of large amounts of sodium to a diet poor in potassium results in a more rapid loss of potassium (Gamble, 1947; Burnett, Burrows and Commons, 1949; Howard and Carey, 1949). It is further known that DCA increases potassium excretion (Regan, Ferrebee, Phyfe, Atchley and Loeb, 1940; Ferrebee, Parker, Carnes, Gerity, Atchley and Loeb, 1941; Loeb, 1942; Darrow and Miller, 1942). Both factors, DCA plus sodium overdosage, therefore act synergistically toward depleting the body of its potassium. In this respect the first five dietary groups are characteristic. While 11 out of 12 high sodium intake animals (3 accidental deaths not included) died, all showing the typical cardiac lesions of severe hypopotassemia, only 3 out of 10 tap water animals died, likewise showing the cardiac changes of potassium deficiency. Of these three, 2 were in the fat cheese group with the lowest potassium intake.

Fifty-nine mg. of potassium daily are apparently insufficient to prevent the anatomic manifestations of potassium deficiency under the conditions

of this experiment. Diets with a higher potassium content (Table 1, groups 6, 7, 8) are adequate to maintain the animals in equilibrium. Kornberg and Endicott (1946) in their low potassium diet experiments determined the minimum daily potassium intake necessary to prevent myocardial lesions. They found it to be 12.6 mg. per rat. It is obvious that this amount is too small as indicated above for the conditions of "stress" imposed by the DCA and sodium overdosage of the present experiment.

The cardiac lesion of severe hypopotassemia is well known. Selye (Selye and Hall, 1943; Selye and Dosne, 1940) tried to rule out this etiologic factor by giving potassium chloride additions to dogs and rats which had been treated with overdosage of DCA and sodium chloride and showed the toxic manifestations characteristic of their species. It has been demonstrated (Smith, Black-Schaffer and Lasater, 1950) that dogs in severe hypopotassemia brought about by special depletion diets develop a flaccid paralysis of the skeletal muscle but never myocardial necrosis. The rat in contrast develops myocardial necrosis but never recognizable skeletal muscle changes. The canine paralysis is completely reversible when adequate amounts of potassium are administered (Ruegamer, Elvehjem and Hart, 1946; Smith, Black-Schaffer and Lasater, 1950). Selye (Selye and Hall, 1943) gave the two dogs with the typical paralysis, produced by DCA overdosage and high sodium intake, 20 cc. of a 2% solution of potassium chloride. This corresponds to 154 mg. potassium chloride per kg. of body weight. Since this was without any noticeable effect, he concluded that the paralysis which is analogous to the myocardial necrosis of the rat, was not due to potassium deficiency. Smith (1946) has found that dogs with potassium deficiency following potassium restriction in the diet could always be cured of paralysis, and death prevented by a dose of 200 mg. potassium chloride per kg. of body weight, but never by 100 mg. per kg. Selye's dose lies between these two figures and is probably inadequate under the conditions of "stress" of his experiment. Also in the case of rats, Selye (Selye, Mintzberg and Rowley, 1945) did not reach the conclusion that the damaging effect of DCA upon cardiac structure might be secondary to its blood potassium depressing effect. He states that the administration of potassium chloride is ineffective to antagonize the actions of DCA in the rat, and that the decrease in the potassium content of the serum caused by DCA cannot be made responsible for the morphologic manifestations of DCA overdosage. But comparing, in his table, the group which received sodium chloride alone, with the group which received potassium chloride in addition, one notices a reduction in the incidence of cardiac lesions from 100% in the former to 17% in the latter, and a reduction in the intensity of the lesions found from 55% to 8%.

The same interpretation of the heart lesions occurring in DCA-treated

animals is contained in Selye's recent book "Stress" (1950). The lesions are again considered as resembling those of rheumatic fever, and rheumatic fever is therefore considered to be a manifestation of the adaptation syndrome. Selye emphasizes again that the changes caused by DCA are not prevented by the feeding of excess potassium.

Since the question of the etiology of rheumatic fever was raised by Selye and his coworkers, it is necessary to point out that the Aschoff nodule of rheumatic fever is a lesion appearing in the interstices of the heart (Figure 1D), centering about a fibrinoid change of the collagen. The myocardial fibers are little if at all involved by this lesion in its early manifestations. The nodule is fairly well circumscribed and composed of Anitschkow cells as well as giant cells (Aschoff cells) whose nuclei retain the characteristic chromatin distribution of the Anitschkow cell. Since the Anitschkow cell is not pathognomonic (Zak, 1947), the abundant presence of these cells alone in the necrotic heart of DCA overdosed rats is no proof of the presence of rheumatic fever.

SUMMARY

Myocardial necrosis was found in rats in which potassium depletion had been caused by low potassium diets together with DCA overdosage with or without high sodium intake.

Both the rapidity with which death occurred, and the number of deaths were inversely proportional to the potassium consumption.

Myocardial lesions and death did not occur if sufficient amounts of potassium had been added.

These findings do not support the view that the heart lesions found in DCA overdosed rats have any connection with rheumatic fever or the adaptation syndrome. The pathologic anatomic studies showed that the myocardial lesion is different from that of rheumatic fever and identical with that produced by potassium exclusion diets.

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A SYNOVIAL FLUID CLOT PREVENTION TEST FOR THE DETERMINATION OF HYALURONIDASE ACTIVITY

GEORGE H. WARREN AND JANE G. DURSO

From the Wyeth Institute of Applied Biochemistry, Philadelphia, Pennsylvania

BURNET and Stone (1947) and Burnet (1948) showed that a mucinase which depolymerized the mucins of glandular and connective tissue, was present in filtrates of cultures of *Vibrio cholerae*. This observation led to the development of a simple, practical test for the titration of mucinase. It was found that if a mucin solution was colored with Congo red and dropped into acid-alcohol, an easily visible clot formed at the interface. In the absence of mucin the drop flattened out and left only a cloudy patch without a definite margin. Furthermore, work of a preliminary nature indicated the applicability of this test with synovial fluid as the "mucin" substrate for the titration of bacterial hyaluronidases.

The present work was undertaken in an attempt to gain more detailed quantitative information concerning the use of a synovial fluid clot prevention test for the assay of bovine testicular hyaluronidases.

MATERIALS

Substrate. Fresh bovine synovial fluid was obtained from a slaughterhouse in pooled lots and centrifuged at 3000 r.p.m. to remove the cellular debris. The supernatant fluid was dispensed in 10 ml. lots in rubber stoppered bottles and immediately stored in the freezing compartment of the refrigerator (Temp. 25° F.). No evidence of the deterioration of the substrate has been observed after 10 months storage.

Before used in the test, the frozen substrate is thawed and diluted with buffer pH 7.0, to determine the concentration of synovial fluid which will produce a firm "mucin" clot when dropped into acid-alcohol. This titration is necessary to obtain a satisfactory clot and at the same time gives a concentration of substrate which possesses the proper sensitivity for the assay procedure. Usually, a 1:8 dilution of synovial fluid produced a satisfactory clot.

In order to avoid bacterial contamination by repeated handling of the substrate, any one bottle of synovial fluid is used within 24 hours following the thawing procedure.

Buffer. A buffer of the following composition is used as diluent for both substrate and enzyme: 0.1% $CaCl_2$, 0.85% NaCl buffered to pH 7.0 with sodium borate and diluted 1:10.

Congo Red. 1% solution in distilled water.

Acid-alcohol. 1% concentrated HCl in 70% ethyl alcohol.

Enzymes. Bovine testicular hyaluronidase was prepared from beef testes by essentially the methods of Madinaveitia (1941) and Hahn (1943). The activity of the various hyal-

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uronidases employed ranged from 75 TRU/MG to 1200 TRU/MG as evaluated by the assay procedure of Kass and Seastone (1944).

PROCEDURE

A dry hyaluronidase sample previously diluted to 1 mg./ml. with the buffer was maintained at 24°C. for 30 minutes. This stock enzyme solution was further diluted so that a final dilution of 1:1,000,000 was obtained. Varying amounts of this dilution were distributed in duplicate in a series of tubes as indicated in Table 1 and the volume was adjusted with the buffer to 0.25 ml. To each tube was then added 0.25 ml. of a 1:8 dilution of synovial fluid. The tubes were shaken, incubated in a 37°C. water bath

TABLE 1. METHOD USED FOR ASSAY OF HYALURONIDASE ACTIVITY

Unknown hyaluronidase solution: ml. of 1:1,000,000 dilution	0.25	0.20	0.15	0.10	0.05
Buffer diluent, ml.	0	0.05	0.10	0.15	0.20
Synovial fluid substrate 1:8, ml.	0.25	0.25	0.25	0.25	0.25
Results of destruction of synovial fluid clot	−	−	−	±	+
Results of destruction of synovial fluid clot by 1:1,000,000 dilution of reference standard	−	−	±	+	+

Conclusion: Since the hyaluronidase standard is arbitrarily assigned a value of 1unit/mg., then the unknown is .20/.15 or 1.33 units/mg. Should the unknown have been diluted further before setting up the test, the result, of course, must be multiplied by the dilution factor.

for 30 minutes and immersed in an ice bath for 5 minutes. Whenever a large number of assays were performed on any one day, the samples were staggered in order to standardize the time factor between sampling and recording the results. To each tube .05 ml. of a 1% solution of congo red was added and mixed. Two control tubes each containing 0.25 ml. of synovial fluid and both heat inactivated hyaluronidase and buffer were included in the test.

With a capillary pipette one drop from each tube is dropped gently into a petri dish containing 20 ml. of acid-alcohol. The shapes assumed by the drops are recorded as follows: (+), a firm clot; (±) partial disintegration of clot; (−) complete disintegration of clot as evidenced by a diffuse haze (see Fig. 1). We have chosen as the endpoint the highest dilution of enzyme which produced a (−) reaction.

The unit and standard solution. In a previous communication (Warren *et al.* 1948), a turbidimetric hyaluronidase assay was described in which a partially purified hyaluronidase preparation was assigned an arbitrary potency of 1.0 TRU/MG.[1] This preparation was retained as standard. The enzyme activity of an unknown was then expressed in TRU/MG

[1] The enzyme preparation contained 150 turbidity reducing units/mg. as evaluated by the assay method of Kass and Seastone (1944).

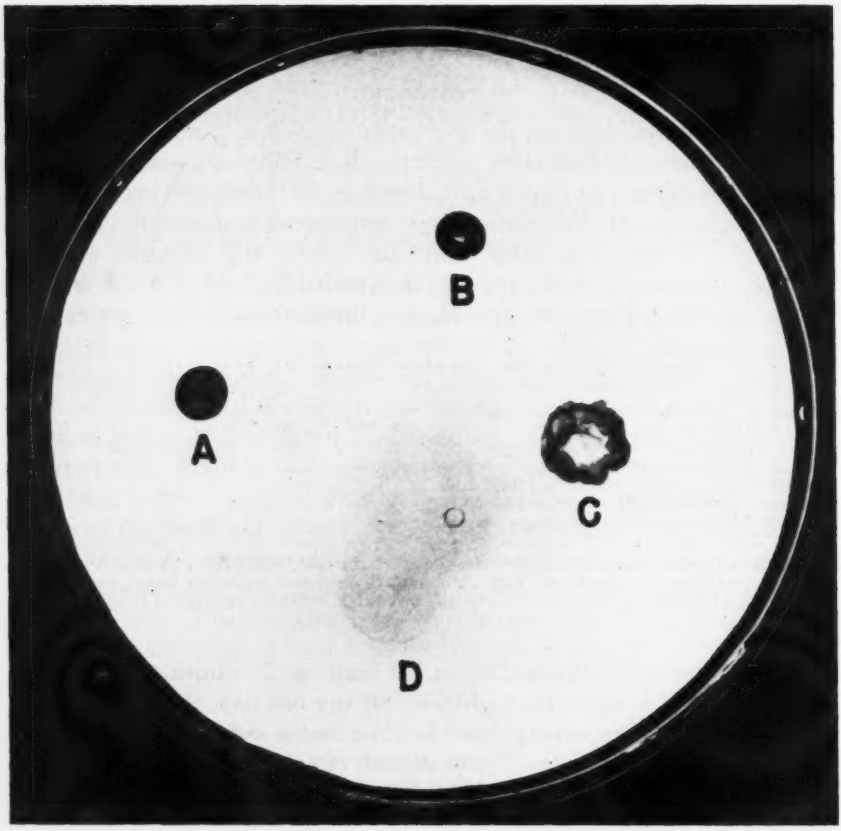

FIG. 1. The depolymerization of synovial fluid hyaluronic acid by bovine testicular hyaluronidase. (A) buffer; (B) heat inactivated hyaluronidase; (C) hyaluronidase (partial depolymerization); (D) hyaluronidase (complete depolymerization).

equivalent of standard. This method for establishing a unit of comparative value has been found to eliminate, for the most part, uncontrollable fluctuations in the assay technique and has been applied as a basis for the synovial fluid clot prevention unit. The method used for estimating the potency of a hyaluronidase sample is illustrated in Table 1.

EXPERIMENTAL RESULTS

In order to determine the accuracy of the test, three preparations of testicular hyaluronidase known to be relatively stable were repeatedly assayed over a period of two months. Several dilutions of the enzymes

ranging from 1:1,000,000 to 1:15,000,000 were employed in the tests. The values presented in Table 2 are based on the activity of a standard which has been arbitrarily assigned a potency of 1.0 synovial fluid clot prevention unit/mg. and which has been discussed previously (see Table 1).

Statistical analyses of these results showed a standard deviation in enzyme units of 0.084 for enzyme A; 0.134 for enzyme B; and 0.61 for enzyme C. The coefficient of variation was found to be 12.4%, 10.2%, and 8.5% for hyaluronidases A, B, and C respectively.

TABLE 2. ASSAYS ON BOVINE TESTICULAR HYALURONIDASES

Experiment No.	Synovial fluid clot prevention units/mg.		Hyaluronidase C*
	Hyaluronidase A*	Hyaluronidase B*	
1	0.75	4.56	7.32
2	0.60	3.98	7.90
3	0.75	4.51	6.73
4	0.60	4.04	7.10
5	0.60	4.20	6.75
6	0.61	5.23	8.75
7	0.63	3.42	6.43
8	0.75	3.45	6.69
9	0.65	3.75	7.44
10	0.90	4.23	7.14
11	0.72	3.92	7.33
12	0.69	4.23	7.48
13	0.65	4.25	7.31
14	0.70	4.10	7.92
15	0.54	4.99	7.64
16	0.62	3.85	6.71
17	0.64	4.33	6.53
18	0.79	4.41	6.33
19	0.61	4.23	8.13
20	0.75	4.20	7.24
Mean	0.68	4.19	7.24
Standard deviation	0.084	0.134	0.61
Coefficient of variation, %	12.4	10.2	8.5

* Average of duplicate tests.

DISCUSSION

An extremely sensitive test for hyaluronidase activity based on Burnet's assay procedure for *V. cholerae* mucinase has been described. Although this test is not so accurate as the turbidimetric methods of assay, it apparently is satisfactory for many purposes and can be performed with greater rapidity than the turbidimetric procedure. Furthermore, it is frequently desirable to employ a hyaluronidase assay technique in which a change in physical character of the substrate may be correlated with enzyme activity.

The synovial fluid clot prevention test is based on the observation that synovial fluid when dropped into acid-alcohol forms a firm clot. After incubation with hyaluronidase, the hyaluronic acid component of the syno-

vial fluid is depolymerized and the synovial fluid is not longer capable of forming a clot. The test is relatively simple, easily readable, and requires a minimum of reagents and apparatus. The substrate may be readily obtained from a slaughterhouse and stored indefinitely at 25°F. Since 0.25 ml. of a 1:8 dilution of synovial fluid is used per tube, there is no problem of maintaining an ample supply of a substrate for the performance of a large number of assays over an extended period of time.

SUMMARY

A synovial fluid clot prevention test for the assay of hyaluronidase is described. The procedure was adapted from Burnet's test for the assay of *V. cholerae* mucinase. The test is simple, rapid and requires a minimum of reagents and apparatus.

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RADIOIODINE CONCENTRATIONS AND CLEARANCES IN RATS RECEIVING IODINE, THYROID AND PROPYLTHIOURACIL

JOSEPH B. BOATMAN AND CAMPBELL MOSES

From the Addison H. Gibson Laboratory of the University of Pittsburgh School of Medicine

THE experiments here reported were conducted to determine the alteration in the distribution and concentration of radioiodine in the pituitary, adrenals, thyroid, muscle and blood of rats maintained on prolonged high iodine intake, on desiccated thyroid and on propylthiouracil and to determine whether these conditions influence the local clearance of I^{131} after intramuscular injection.

METHODS

Five groups of five young, female albino rats (Groups I–V) were given graded doses of Lugol's solution daily in their drinking water over a period of 120 days. Five animals (Group VI) received no Lugol's solution over the same period. Drinking containers were emptied and washed daily and replaced with from 1 to 5 drops of fresh Lugol's solution in 100 ml. of tap water (approximately 0.9 to 4.3 mg. of iodine daily for each rat). Rockland rat ration was provided, supplemented weekly with fresh vegetables.

One colony of young female albino rats was randomly divided into an experimental (Group VII) and a control group (Group VIII) of 12 animals each, and a second group of young male albino rats was similarly divided into an experimental (Group IX) and a control group (Group X) of 6 animals each. The female experimental group received desiccated thyroid for 119 days while the male experimental group received propylthiouracil for 113 days. The female animals were approximately three weeks older than the male animals when dosage was begun. The male group fed propylthiouracil and their controls were sacrificed two weeks after the group fed desiccated thyroid and their control group.

The desiccated thyroid was prepared for administration every second day, and refrigerated except as used. 800 mg. of compressed tablets* were ground and suspended in 1000 ml. of distilled water. The rats were pair fed 100 ml. (40 mg. per rat) daily of the prepared thyroid solution. The desiccated thyroid suspension was shaken well before filling each drinking container. The containers emptied by gravity flow on drinking so that the ground thyroid was taken with a minimum of precipitate remaining in the drinking container.

250 mg. of propylthiouracil† was ground and dissolved in 1000 ml. of distilled water. The treated rats were pair fed 100 ml. (12.5 mg. per rat) of this solution daily. All drink-

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* Sharpe and Dohme, Philadelphia, Pennsylvania.

† Parke, Davis & Co., Detroit, Michigan.

ing containers for all groups were washed each day, and weights were recorded every second day.

Following the method described by Kety (1949) and as modified for use on rats, (Boatman, *et al.*, 1950, Franke, *et al.*, 1950) leg muscle clearances were determined on Groups I–VI 45 days and 76 days after the administration of Lugol's solution was begun; on Group VII, 74 and 100 days from the start of thyroid dosage; Group VIII, 71 and 98 days from the start of thyroid dosage of Group VII; Group IX and X, 52 and 88 days from the start of propylthiouracil dosage of Group IX. Muscle clearance rates were recorded as per cent of injected activity of I^{131}‡ remaining at the site of injection after 5 minutes (5′%AR) and after 10 minutes (10′%AR) (Boatman *et al.*, 1950).

200 microcuries of I^{131} in 1–2 ml. of normal saline were injected intraperitoneally three hours before sacrifice. At sacrifice all animals were anesthetized with ether and heart blood was collected in heparinized centrifuge tubes.

The weight of the liver, thyroid, adrenals, pituitary and a muscle sample were recorded. Weighed portions of liver and muscle, and the entire thyroid, both adrenals, and the pituitary were prepared for radioassay after gentle removal of excess blood.

All blood samples were diluted with distilled water. Aliquots were pipetted into stainless steel planchets and dried at 70° C. Tissue samples were ashed in stainless steel planchets after wetting with 0.1 N·$AgNO_3$. The samples were covered with small aluminum caps and placed in a muffle furnace at 500° C. The aluminum caps were later assayed for radioactivity and all significant activity above normal background was added to the original sample's assay. This proved to be under 7% of the original sample's activity in all cases and less than 10% of the aluminum caps had any demonstrable activity. The original samples were wet with $AgNO_3$ solution again after ashing and dried at 70° C. before assay.

All assays were corrected for radioactive decay. The percentage of the injected solution that was found in the organ was expressed as the per cent activity of the respective tissue.

To determine the relative degree of concentration of radioiodine in an organ the percentage radioactivity of the organ was divided by its per cent of total body weight as reported by Franke (1950). Thus if the concentration of radioiodine were greater or less than that organ's percentage of the body weight, the ratio would be more or less than unity. This is referred to as the concentration ratio.

The sacrifice blood samples were assayed for the percentage of activity of the injected radioiodine in the whole blood, plasma and erythrocyte fractions. The blood volume was estimated as being 5.0 ml. whole blood per 100 grams of body weight (Sharpe, Culbreth and Klein, 1950). Wintrobe hematocrit tubes were used to determine the percentage of plasma and erythrocytes in whole blood.

The determination of total blood water volume was based upon the sum of 65% of the erythrocyte volume and 93% of the plasma volume (Rall, Power and Albert, 1950). The percentage of whole blood radioiodine in the erythrocyte divided by the percentage of blood water volume in the erythrocyte was considered to be the relative degree of radioiodine concentration in the erythrocyte fraction (Boatman and Moses, 1951).

The results were tested for significant differences between the means of each group against their control groups. (p was determined from Fisher's "t.") The dissimilarity in sex, age, weight, and sacrifice time between the control groups prohibited a comparison of data between these groups.

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‡ From the Oak Ridge National Laboratories, Oak Ridge, Tennessee.

RESULTS

I. Feeding of Iodine

The amount of inorganic iodine in the diet showed no significant effect on the rate of blood flow from the muscle as measured by muscle clearance methods using radioiodine (Table 1).

TABLE 1. MUSCLE CLEARANCES OF IODINE FED RATS USING I^{131} IN MEAN PERCENTAGE ACTIVITY REMAINING (%AR) LOCALLY 5 AND 10 MINUTES AFTER INJECTION

Group	Daily I_2 intake, mgs.	%AR after 45 (a) and 76 (b) days	
		5′	10′
I	0.9	a 47.6 b 42.2	31.7 29.2
II	1.7	a 53.4 b 49.5	45.1 28.4
III	2.6	a 68.7 b 61.2	59.3 42.4
IV	3.4	a 45.5 b 55.8	31.2 37.8
V	4.3	a 67.2 b 48.5	50.0 38.8
VI	0.0	a 51.5 b 43.0	35.1 30.5

It has been reported by Hanzlik, Tabot and Gibson (1928) that the continued feeding of iodine to rats in small daily doses over long periods caused moderate though variable increases in weight and growth of the body. The iodine supplied to the rats in this study (Groups I–V) was estimated to be 10 to 200 times that of a well balanced diet for rats (McCoy, 1949). The percentage of body weight of the adrenals and pituitary were lower in the iodine fed groups, and those groups that on sacrifice had significantly higher radioiodine pituitary concentration ratios also had significant weight increases (Tables 2 and 3).

The adrenals showed a significant increase in their mean concentration ratio from the control group with increased intake of iodine. The depression of the thyroid's concentration of radioiodine was noted to significantly increase with increased amounts of Lugol's solution until the group receiving the highest dosage was noted to have less than 10% of the concentration ratio of radioiodine of the control group. The muscle radioiodine concentration ratio also declined significantly with increasing dosages of iodine (Table 3).

Three hours after the injection of radioiodine the whole blood of the treated animals held a greater quantity of radioiodine than did the control

TABLE 2. ORGAN WEIGHT CHANGES IN TERMS OF BODY WEIGHT OF IODINE FED RATS

Group		Thyroid	Pituitary	Adrenal	Liver	Mean cumulative body wt. increase
I	Mean weight in mgs.	(A) 21.5	15.5	54.4	7.168	139.4 gms.
	Mean % body weight	(B) .010	.007	.024	3.200	
II		(A) 28.6	14.7	36.2	11.440	248.2 gms.*
		(B) .008	.004*	.010†	3.186	
III		24.1	11.7	32.6	8.559	223.8 gms.†
		.008	.004†	.011†	2.892	
IV		20.4	9.9	36.2	7.155	152.2 gms.
		.009	.004*	.024	3.152	
V		20.3	8.6	33.8	7.330	231.2 gms.†
		.007	.003†	.011†	2.460	
VI		19.3	14.6	52.7	7.673	155.5 gms.
		.008	.006	.023	3.294	

* Significant difference from Control Group VI with "p" less than 0.05.
† Significant difference from Control Group VI with "p" less than 0.01.

group, the plasma contained about the same, while the erythrocytes were responsible for the major portion of the increase of whole blood radioiodine (Table 4).

A greater percentage of the whole blood radioiodine was in the erythrocyte than the percentage of whole blood water in the erythrocyte, which was not the case in the control animals (Table 5). The substantial increase of radioiodine content in the erythrocyte under high iodine feeding represents a concentration 1.5 times the water concentration of the erythrocytes as opposed to a nearly equal percentage concentration of both in the normal control group. The values found for the control animals (Group VI) in

TABLE 3. CONCENTRATION RATIOS[1] OF I^{131} IN VARIOUS TISSUES OF IODINE FED RATS

Group	Thyroid	Adrenals	Liver	Muscle (1 gm.)	Pituitary	Body weight increase ratio[2]
I	48.990	.079	.269	.171	.420	2.412
II	13.089*	.620†	.422	.260	1.150*	3.421†
III	24.321*	.366†	.465	.100*	1.285*	4.174†
IV	10.440*	.086	.252	.138*	.269	2.745
V	5.059†	.265†	.466	.086†	.821*	4.072*
VI	54.000	.168	.224	.350	.452	3.039

* Significant difference from Control Group VI with "p" less than 0.05.
† Significant difference from Control Group VI with "p" less than 0.01.

[1] Concentration Ratio $= \frac{\text{Per cent of total Administered Radioactivity in Organ}}{\text{Organ's Per Cent of Body Weight}}$.

[2] Body Weight Increase Ratios $= \frac{\text{Final Mean Weight}}{\text{Initial Mean Weight}}$.

TABLE 4. I^{131} DISTRIBUTION IN BLOOD OF IODINE FED RATS THREE HOURS AFTER INTRAPERITONEAL ADMINISTRATION

Group	Hematocrit	Whole blood I^{131}	Plasma I^{131}	Erythrocyte I^{131} (by difference)
I	47.7%	26.2%*	0.3%	25.9%*
II	42.9	6.6*	2.9	3.7†
III	46.3	6.0	4.5	1.5
IV	44.5	6.1	3.4	2.7*
V	52.1	11.7†	6.8	4.9†
Mean (I–V)		11.3	3.6	7.7
VI	45.8	5.4	3.4	2.0

* Significant difference from Control Group VI with "p" less than 0.10.
† Significant difference from Control Group VI with "p" less than 0.01.

this study correspond very closely to those given in an earlier report (Boatman and Moses, 1950).

Through all the data it was noted that there was a "peaking" effect with the dosage level of Group III rats. This group had the greatest increase of weight during the study, the slowest muscle clearance, a markedly different blood radioiodine distribution, the lowest erythrocyte percentage of total administered radioiodine, the greatest increase of body weight and the highest pituitary concentration ratios.

II. Feeding of Desiccated Thyroid

An acceleration of growth was shown in those animals receiving desiccated thyroid (Group VII), represented by a weight increase factor of 1.532 with a mean cumulative gain in weight of 76.5 grams as opposed to the control group with a weight increase factor of 1.252 and a mean cumulative gain in weight of 40.9 grams, which was significant to a probability of less than 0.01 (Fig. 1).

No significant changes in muscle clearances of I^{131} were noted in the thyroid fed group, compared to their controls (Group VIII) (Table 6). The distribution of blood radioiodine at sacrifice showed a significantly

TABLE 5. PERCENTAGE OF WHOLE BLOOD RADIOIODINE ACTIVITY FOUND AFTER THREE HOURS IN ERYTHROCYTES OF IODINE FED RATS IN TERMS OF WHOLE BLOOD WATER CONTENT

Group	Percentage whole blood I^{131} in erythrocyte	Percentage whole blood water in erythrocyte	Ratio
I	98.83%	39.0%	2.55
II	56.65	34.4	1.92
III	24.88	37.5	.66
IV	43.78	35.9	1.22
V	41.71	43.2	.97
Mean (I–V)	50.97	38.0	1.47
VI	36.68	37.2	.99

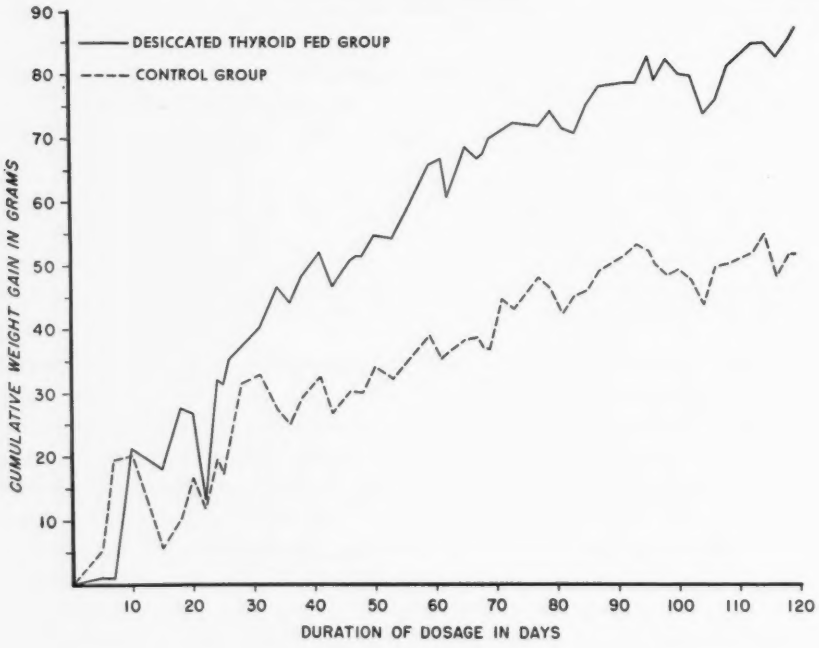

FIG. 1

greater percentage in the erythrocytes of rats fed thyroid than their control group (Table 7).

TABLE 6. MUSCLE CLEARANCES IN THYROID AND PROPYLTHIOURACIL FED RATS USING I^{131} IN TERMS OF MEAN PERCENTAGE ACTIVITY REMAINING AT 5 MINUTES (5′%AR) AND 10 MINUTES (10′%AR)

Thyroid fed group		Control group		Propylthiouracil group		Control group	
(79th day)	(100th day)	(71st day)	(98th day)	(52nd day)	(88th day)	(52nd day)	(88th day)
5′%AR							
63.5	56.7	65.1	63.3	40.0	62.2†	47.3	48.1
10′%AR							
41.2	40.0	49.2	44.7	24.0	45.3*	29.8	30.6

* Significant difference from Control Group with "p" less than 0.05.
† Significant difference from Control Group with "p" less than 0.02.

Thyroid feeding increased the concentration ratio of radioiodine in the adrenals and pituitary, and depressed it in the thyroid gland (Table 8). The percentages of body weight of the adrenals, pituitary and liver were increased with the prolonged feeding of desiccated thyroid.

TABLE 7. DISTRIBUTION OF INJECTED RADIOIODINE IN THE BLOOD OF THYROID AND PROPYLTHIOURACIL FED RATS

	Erythrocyte			Plasma		
	Whole blood I^{131}	Whole blood water	Conc. ratio	Whole blood I^{131}	Whole blood water	Conc. ratio
Propyl Thiouracil fed	41.3*	32.06	1.288	58.7*	67.93	.8567
Control	50.0	44.97	1.112	50.0	55.02	.9087
Thyroid fed	62.6*	41.91	1.494	31.8*	58.08	.5475
Control	31.8	41.82	.7604	68.2	58.17	1.172

* Significant difference from Control Group with "p" less than 0.10.

III. Feeding of Propylthiouracil

The animals fed propylthiouracil showed a marked depression of growth, the weights of the animals increasing only by a factor of 1.219 compared to that of their controls of 1.765 (Fig. 2). Cumulative gains in weight were

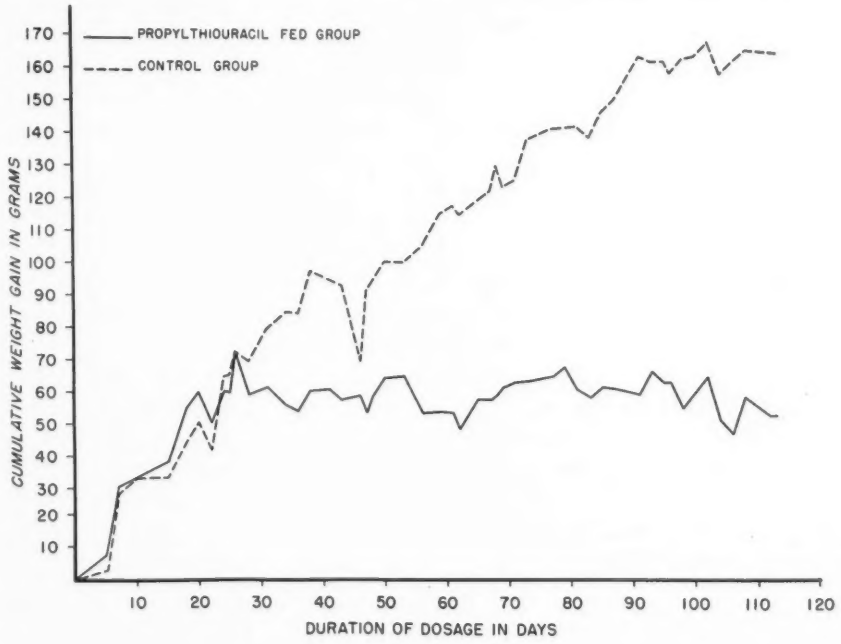

FIG. 2

40.2 and 153.0 grams, respectively, significant to a probability of less than 0.01.

Muscle clearance determinations 52 days after the beginning of propylthiouracil dosage showed no change from the control animals (Table 6).

36 days later the propylthiouracil treated animals showed a mean retention of 25.2% more I^{131} at the muscle injection site after 5 minutes than the earlier determinations, and 24.1% more after 10 minutes (significant to a probability of 0.02). The control group showed no significant change in muscle clearance during this period.

The animals fed propylthiouracil showed a lower percentage of radioiodine in the erythrocytes than their controls (Table 7), and a depression of the concentration ratio of radioiodine in the thyroid, liver, muscle, adrenals, and pituitary (Table 8).

TABLE 8. ORGAN WEIGHTS AND RADIOACTIVITY OF THYROID AND PROPYLTHIOURACIL FED RATS

	Thyroid treated			Control		
	% body wt.	% administered radioactivity	Radioactivity concentration ratio	% body wt.	% administered radioactivity	Radioactivity concentration ratio
Adrenal	.030†	.038	1.263†	.025	.009	.364
Pituitary	.009*	.041	4.958†	.007	.010	1.412
Thyroid	.008	.307	36.615*	.008	.461	55.342
Liver	3.604†	.735	.204	2.755	.695	.252
Muscle	.449	.052	.115	.465	.082	.177
	Propylthiouracil treated			Control		
Adrenal	.012	.007	.510*	.010	.008	.739
Pituitary	.009†	.012	1.323*	.004	.016	3.853
Thyroid	.026†	.010	.396†	.007	.776	113.260
Liver	3.137	.285	.091†	2.685	.846	.315
Muscle	.454	.018	.040*	.282	.029	.104

* Significant with "p" less than 0.05.
† Significant with "p" less than 0.01.

CONCLUSIONS

Muscle clearance measurements of the rate of blood flow with radioiodine are not distorted by the presence of large dietary levels of stable iodine. On the basis of data from this study, the muscle clearances reflect a normal blood flow in the extremities of rats on a high iodine diet.

The muscle clearance of radioiodine is decreased in rats fed propylthiouracil, while those fed desiccated thyroid showed no significant changes. A single clearance value may be misleading as in the propylthiouracil fed rats. The initial 5 minute clearances showed a mean rate of removal slightly faster than the control group. Thirty-six days later the iodine clearance had decreased and 25% more I^{131} remained after 5 minutes, while the clearance vaules of control animals remained unchanged.

It is seen that both the adrenal and pituitary concentration of radioiodine are increased with high iodine intake and with the feeding of thyroid while the thyroid concentration of radioiodine is depressed with both diets.

However, the percentage of body weight of the adrenals and the pituitary were depressed with iodine feeding and were increased with thyroid feeding.

Williams *et al.* 1944 reported the distribution of thiouracil throughout all the tissues and fluids of the body with a relatively high concentration in the pituitary, adrenal, thyroid, ovary and bone marrow. The dosing of rats with propylthiouracil produced a reversal of the effects seen with the iodine and thyroid dosage, resulting in a depression of concentration of radioiodine in all tissues.

SUMMARY

Muscle clearances of I^{131} in rats treated with Lugol's solution for 120 days were found to be within the normal range compared with control animals and it was concluded that measurement of muscle clearance of I^{131} is not distorted by the presence of large dietary intake of stable iodine. Rats maintained for 120 days on propylthiouracil showed a significant depression of muscle clearance of I^{131} and no significant change with desiccated thyroid.

Radioiodine content of whole blood after three hours was higher in the iodine fed rats than in the blood of control animals. This increase was almost entirely due to an increase of erythrocyte radioiodine. A greater percentage of blood I^{131} was found in the erythrocytes of thyroid fed rats, while those fed propylthiouracil showed the same or slightly less percentage of blood I^{131} in the erythrocytes than did their control group.

Thyroid and muscle radioiodine concentration ratios (organs % injected activity/organs % body wt.) were significantly depressed with increased dosage levels of iodine, while the concentration ratio of the liver remained approximately that of the control animals, and the adrenals and pituitary showed significantly increased concentration ratios. Thyroid fed rats showed an increased concentration ratio of I^{131} in the adrenals, pituitary, and thyroid, while propylthiouracil fed rats showed a decreased concentration ratio in adrenals, pituitary, thyroid, liver and muscle, compared to control animals.

The feeding of iodine decreased the pituitary and adrenal percentages of body weight. Thyroid fed rats showed increased percentage of body weight of the adrenals, pituitary and liver, and propylthiouracil fed rats showed increased percentages of body weight of the pituitary and thyroid, compared to control animals.

Body weight increase was augmented with desiccated thyroid feeding and depressed with the feeding of propylthiouracil over the period of dosage. Significantly higher pituitary radioiodine concentration ratios were found in those groups fed Lugol's solution with significant weight increases.

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THE EFFECTS OF GROWTH HORMONE ON GLYCOGEN IN TISSUES OF THE RAT[1]

BARBARA A. ILLINGWORTH[2] AND JANE A. RUSSELL[3]

From the Department of Physiological Chemistry, Yale University School of Medicine, New Haven

EXTRACTS of cattle pituitaries and more recently purified growth hormone have been shown to maintain normal glycogen levels in the gastrocnemius muscles of fasting hypophysectomized rats, an action which has been termed the glycostatic effect (Russell, 1938a; Russell and Wilhelmi, 1950). In normal rats fed glucose, such hormone preparations given acutely also increase the deposition of glycogen in the muscle (Russell, 1938b; Milman and Russell, 1950). The minimum effective dose of growth hormone given to hypophysectomized rats during the 24 hour fast was found to be about 100–200 μg. per 100 gm. A similar order of dosage has now been found to effect an increase in carbohydrate storage in the fed normal animal. Observations on the effects of growth hormone *in vivo* have been extended to include data on changes in heart, diaphragm, and liver glycogen in normal rats, both fasting and fed glucose, and in fasting hypophysectomized rats. The actions of the growth hormone in adrenalectomized and in diabetic animals also have been studied, and in normal rats comparisons have been made between the actions of growth hormone and of insulin.

METHODS

The animals used were male albino rats of the Sprague-Dawley strain, 2 to 3 months of age and weighing 200 to 300 gm. The diet was Purina Laboratory Chow, fed *ad libitum*. In most of the experiments two types of observations were made: In the first, the animals were fasted 18 to 20 hours, given a single injection of growth hormone, and the fast prolonged another 6 hours before analysis. In the other series, the animals were fasted and treated with growth hormone as described; then 2 hours later they were fed glucose and were sacrificed after another 4 hours. The amounts of glucose given, usually

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[2] Public Health Service Research Fellow, 1948–1950. Present address: Department of Biochemistry, Washington University School of Medicine, St. Louis, Missouri.

[3] Present address: Department of Biochemistry, Emory University, Georgia.

5 ml. of a 50 per cent solution, or to adrenalectomized rats, 3 or 4 ml., were sufficient to provide for maximal absorption from the intestinal tract during the 4-hour period. In this way the effects of the growth hormone on the fasting glycogen reserves and on the deposition of fed carbohydrate were estimated. Control observations were made in an identical manner except that the animals were given saline solution instead of growth hormone, or in some cases were left untreated; no effects of the saline injections could be discerned. Usually three experimental animals were observed concurrently, the group in each case including both control and experimental animals. All samples were taken between 3:00 and 4:00 P.M.

The growth hormone used was prepared by the method of Wilhelmi, Fishman, and Russell (1948). Seven different crystalline preparations were used, all homogeneous as judged by electrophoretic analysis. Assays for adrenocorticotrophic hormone by the method of Sayers *et al.* (1948) carried out on several of the preparations indicated no detectable contamination with this hormone. Immediately before use, the material was suspended in 0.9 per cent saline and dissolved completely by the addition of a very small amount of alkali. All injections were made intraperitoneally in a volume of 0.25–0.50 ml.

At the end of the experimental period (usually six hours after the administration of hormone), the rats were anesthetized with sodium pentobarbital and the tissue samples removed in the following order: both gastrocnemii, the heart, the diaphragm, and the the liver. For the muscle samples, the whole organs were taken, for the liver 2 to 3 gm. of tissue. In most instances the two gastrocnemii were analyzed separately and the mean taken as a single observation. If the second gastrocnemius was removed after the other samples, the agreement between the glycogen contents of the two muscle samples was poor. As others have found (see Evans, 1934), the heart must be removed extremely rapidly if large losses of glycogen are to be avoided. In these operations, the abdomen was opened with a midline incision, the xiphisternum elevated, the rib cage opened with a single slash of the scissors, the heart grasped in the operator's fingers, and the main blood vessels severed also with a single cut. No changes were observed in the diaphragm or liver samples taken at different times during the sampling period. All tissues immediately on removal were placed in tared tubes containing 30 per cent potassium hydroxide (1 to 2 ml. per gm. of tissue). The tubes were then re-weighed quickly and placed in boiling water. The time elapsing between the first incision and placement of the last sample in the water bath was about 5 minutes.

Glycogen was determined by the anthrone reaction (Morris, 1948; Viles and Silverman, 1949; Seifter *et al.*, 1950). Initial experiments were conducted to determine the effects of changing the concentration of anthrone in the reagent and of sulfuric acid in the reaction mixture; the optimum conditions for mixing reagent and sample; the wave length of maximum light absorption; and the effects of hydrolysis of the glycogen. The conditions for color development finally adopted were essentially those described by Morris. Glycogen samples gave the same color before and after hydrolysis. Since the amounts of glycogen present in most of the samples were rather small, the glycogen was routinely precipitated from the alkaline digest by the addition of 2 volumes of alcohol. After the alcoholic mixture was heated to boiling, cooled, and allowed to stand overnight, the glycogen was separated by centrifugation, dissolved in water, and an aliquot taken for color development with the anthrone reagent. The usual sample size was about 100 μg. as glucose. This was made up to 4 ml. with water and the color developed by the rapid addition of 8 ml. of the anthrone reagent. After the tubes were cooled in air, readings were made in a Coleman Junior Spectrophotometer at 625 mμ

(the absorption maximum) or in some instances at 540 mμ. Samples of purified glycogen carried through the precipitation procedure alone or added to the alkaline digest were recovered quantitatively. Where the two gastrocnemius muscles were taken, or two samples of liver were analyzed, the agreement between duplicates was usually within 3 per cent.[4] The anthrone method was used also for the estimation of urine glucose. Blood glucose was determined on copper tungstate filtrates with the phosphate buffered reagent of Somogyi (1945), and the arsenomolybdate color reagent of Nelson (1944).

Adrenalectomized animals, operated by the lumbar approach under ether anesthesia, were given saline drinking fluid during the interval between operation and experiment (10 to 16 days). Hypophysectomy was performed by the usual parapharyngeal approach and the completeness of the operation was checked at autopsy. Experimental diabetes was produced by the intravenous injection of alloxan, usually 40 mg. per kg. in 2 per cent solution. Although the results of this treatment were more consistent than when intraperitoneal injections of alloxan were made in fasted animals, there was still considerable variation in the number of animals which died, became diabetic, or were unaffected. The severity of the diabetic state in individual animals was judged by the blood sugar levels in the fed and fasted states and by the amounts of glucose excreted daily. The observations reported here were made on animals which were excreting several grams of glucose daily some weeks after the administration of alloxan.

RESULTS

Fasting normal and hypophysectomized animals. The administration of 1–2 mg. of growth hormone per 100 gm. body weight to fasting normal rats and prolongation of the fast an additional six hours resulted in an increase in the amount of glycogen found in all four of the tissues examined (Table 1). The total increase in glycogen in the body over that seen in untreated fasted animals, as calculated from the conventional percentages (i.e., the gastrocnemius glycogen representing muscle equivalent to 50 per cent of the body weight, etc.) amounted approximately to 8 mg. per 100 gm. per hour. Since growth hormone diminishes nitrogen excretion, it is unlikely that this accumulation could have resulted from increased gluconeogenesis from protein. Such a quantity is considerably less than the rate of new formation of carbohydrate postulated from other experiments to occur in the normal fasting rat: The amount of glucose which must be infused to maintain the blood sugar level in the eviscerated rat, which may be taken as a measure of the normal rate of gluconeogenesis, has been estimated at 15 to 20 mg. per 100 gm. per hour (Frame and Russell, 1946; Ingle and Nezamis, 1949); while the nitrogen excretion of fasting rats, 3–4 mg. per 100 gm. per hour, would indicate also gluconeogenesis from protein of about the same magnitude. Thus the carbohydrate accumulated in the growth hormone treated fasting rats could be provided by a normal or retarded rate of gluconeogenesis if the utilization of carbohy-

[4] Root mean square of differences between duplicates as per cent of mean of the series.

drate by means other than glycogen formation were diminished. No effects of the growth hormone or pituitary extracts on the R.Q. of fasting normal rats have been noted, but the diminution in carbohydrate utilization indicated would be so small compared to the total caloric output that it probably could not be detected by the usual respiratory measurements. In fasting hypophysectomized rats, pituitary extract has been observed to decrease the high R.Q. characteristic of this condition (Fisher, Russell, and Cori, 1936).

As shown earlier, the hypophysectomized rat on fasting suffers abnormal depletion of the gastrocnemius glycogen (Russell and Bennett, 1937), and growth hormone given in divided doses during a 24-hour fast can bring

TABLE 1. THE EFFECTS OF GROWTH HORMONE AND INSULIN ON GLYCOGEN LEVELS IN FASTING NORMAL AND HYPOPHYSECTOMIZED RATS

	No. of animals	Glycogen (mg. per cent)			
		Gastroc. muscle	Heart	Diaphragm	Liver
A. Normal rats					
1. Untreated	10	550 ±18*	672 ±23	281 ±12	91 ±5
2. Growth hormone (1–2 mg. per 100 gm., 6 hrs. before)	11	631† ±21	863† ±58	382† ±11	230† ±31
3. Insulin (0.1 unit per Kg., 6 hrs. before)	9	507 ±13	672 ±22	751† ±47	140 ±21
B. Hypophysectomized rats					
1. Untreated	10	306 ±18	433 ±19	204 ±14	66 (4)
2. Growth hormone (0.25–0.35 mg. per 100 gm. during 24 hours)	8	451† ±18	580† ±25	336† ±19	56 ±10

* Standard error of the mean.
† p (for difference from corresponding untreated group) <.01.

about maintenance of normal concentrations of glycogen in this tissue (Russell and Wilhelmi, 1950). Observations of a similar nature now have been made on other muscle tissues, namely the heart and diaphragm (Table 1). In the untreated fasting hypophysectomized rat, the glycogen levels of these tissues, like that of the gastrocnemius, were lower than normal. On treatment with growth hormone, 250–350 μg. per 100 gm. of body weight given in 3 doses during the fasting period (not only in the last 6 hours as in the experiments reported above), cardiac and skeletal muscle glycogen values were increased, although not quite to normal, and the diaphragm values held above normal. The animals used here had been hypophysectomized at an early age (100 gm.), and as mentioned in the previous paper, such animals appear to require more hormone given at more frequent intervals than do larger animals for the complete mainte-

nance of gastrocnemius glycogen. Possibly a larger dose of growth hormone would have restored completely the glycogen content of the heart and leg muscle as well as that of diaphragm.

Normal animals fed glucose. When fasting normal rats were treated with growth hormone 2 hours before being fed glucose, more glycogen was accumulated in all of the muscle tissues examined than was formed in the tissues of untreated control animals (Table 2). Similar effects were noted

TABLE 2. THE EFFECTS OF GROWTH HORMONE AND INSULIN ON GLYCOGEN LEVELS IN NORMAL RATS FED GLUCOSE

	No. of animals	Glycogen (mg. per cent)			
		Gastroc. muscle	Heart	Diaphragm	Liver
1. Untreated	11	777 ±35	775 ± 43	1338 ± 67	3470 ±240
2. Growth hormone					
a. 1–2 mg. per 100 gm.	17	836* ±23	1017‡ ± 38	1553† ± 62	2850 ±190
b. 0.2–0.5 mg. per 100 gm.	8	876† ±29	1037‡ ± 63	1605† ± 88	3360 ±110
c. 0.1 mg. per 100 gm.	11	856* ±35	966‡ ± 59	1520* ± 87	3530 ±230
All treated animals	36	851 ±16	1009 ± 28	1554 ± 42	3170 ±120
Increase over untreated§		±74† ±35	±234‡ ± 64	±216‡ ± 84	−300 ±256
3. Insulin 0.1 unit per kg.	8	754 ±30	767 ± 24	1723‡ ±126	3330 ±280
4. Growth hormone (1mg. per 100 gm.) and Insulin	7	770 ±28	940‡ ± 34	1570† ± 87	3780 ±240

* $p = .10–.20$ for difference from untreated group.
† $p < .05$ for difference from untreated group.
‡ $p. < .01$ for difference from untreated group.
§ The p values here were obtained both by analysis of variance for 3 treated groups vs. control group and by comparison of all treated animals with the untreated group.

whether the dose was 1 or 2 mg. per 100 gm., or reduced to 0.1 mg. per 100 gm. All of the purified growth hormone samples tested were active at the latter dose, and some appeared to be effective at 0.05 mg. The order of the increase in glycogen storage in treated animals (over that seen in the respective untreated groups) tended on the average to be greater in the rats fed glucose than in the fasting animals, but the difference appeared significant only in the diaphragm. It would have been of interest to find whether the minimum effective dose of growth hormone in normal rats was of the same order in the fasting as in the fed state; from early experiments with crude anterior lobe extracts, where no effect on the muscle glycogen of normal fasting rats but large increases in glycogen of animals fed glucose

were seen (Russell, 1938b), it might be expected that the fed animals would prove to be more sensitive to the hormone.

Adrenalectomized animals. In fasting adrenalectomized rats maintained with salt, the gastrocnemius glycogen was normal, the cardiac glycogen somewhat diminished (observed also by Evans, 1941), and that of the diaphragm elevated (Table 3). The effects of fasting on the carbohydrate stores of adrenalectomized rats thus were quite different from those in

TABLE 3. THE EFFECTS OF GROWTH HORMONE ON GLYCOGEN LEVELS IN ADRENALECTOMIZED RATS

	No. of animals	Glycogen (mg. per cent)			
		Gastroc. muscle	Heart	Diaphragm	Liver
A. Fasted rats					
1. Untreated	9	512 ±17	521 ±15	442 ±16	92 ± 5
2. Growth hormone*	7	565‡ ±19	532 ±22	487‡ ±18	88 ± 5
3. Growth hormone* and A.C.E.†	6	536 ±24	528 ±40	422 ±19	128 ± 18
B. Fed glucose					
1. Untreated	5	680 ±30	486 ±40	993 ±50	1620 ±440
2. Growth hormone*	5	649 ± 9	451 ±35	1013 ±97	2690 ±240
3. A.C.E.†	8	700 ±21	542 ±25	1008 ±81	2870 ±330
4. Growth hormone* and A.C.E.†	7	757§ ±30	678§ ±22	1170‖ ±48	2450 ±310

* 1–2 mg. per 100 gm.
† Aqueous adrenocortical extract (Wilson), 0.2 ml. per 100 gm.
‡ p for difference from untreated groups $<.05$.
§ By analysis of variance, p (of chance difference from 3 other groups fed glucose) $<.01$.
‖ By analysis of variance, p (of chance difference from 3 other groups fed glucose) $=.06$.

evidence after hypophysectomy. In the absence of the adrenals, growth hormone, given either alone or with small amounts of cortical extract, was relatively ineffectual in altering the glycogen stores of the muscles as observed 6 hours later. (A slight increase in the glycogen of the leg muscle and of the diaphragm may have occurred in the animals given only growth hormone.) Conceivably, this failure may have been a consequence of subnormal rates of gluconeogenesis in these animals.

When adrenalectomized rats were fed glucose, the amounts of glycogen deposited in the muscle tissues and in the liver all were rather less than in the normal rats, probably because of some diminution in the ability to absorb the concentrated glucose solutions fed. Growth hormone given before the glucose feeding failed to alter the deposition of glycogen. However,

when a small amount of adrenocortical extract (0.5 ml. Wilson's aqueous extract per animal) was given along with the growth hormone, consistent increases in the glycogen of the muscle tissues were seen. This amount of cortical extract by itself had no effect on the muscles examined. The significance of the synergistic action for each tissue was verified by an analysis of variance, in which the glycogen values in the doubly treated animals were shown to differ from those of the other three groups in each instance (Table 3). The cortical hormone appeared to increase somewhat the deposition of liver glycogen. Data not shown in the table indicated that this amount of cortical extract either alone or in combination with growth hormone had no effect on the deposition of glycogen in the liver or muscles of normal rats.

Effects of insulin and diabetes. In view of the established action of insulin in augmenting glycogen deposition in muscle, and of indications that growth hormone may bring about some increase in the secretion of insulin in fasting rats (Milman and Russell, 1950), the question arises whether the action of growth hormone in these experiments may have been mediated by insulin. Hence, parallel observations were made after the administration of insulin in a dose such that the hypoglycemia induced was of the same order as that seen after the larger amounts of growth hormone. The dose of insulin employed was 0.1 unit per kg. of zinc insulin, given six hours before the tissues were taken for analysis. The maximum fall in the blood sugar in the fasted animals (from samples taken at $\frac{1}{2}$, 2, 4 and 6 hours) averaged 16 mg. per cent. Larger doses of insulin could not be given if the glycogenolytic action of epinephrine were to be avoided. As shown in Tables 1 and 2, the administration of insulin was followed by a notable elevation of the glycogen content of the diaphragm both in the fasting animals and in those fed glucose, but no other changes in glycogen concentrations were seen. In the diaphragm, the increase in glycogen deposition after feeding, over that seen in the corresponding fasting groups, was less in the insulin treated than in either the control or growth hormone treated animals. When growth hormone was given with the insulin before glucose was fed, the effect of the former hormone on the heart glycogen persisted. It is possible that there was some antagonism between the two hormones in their action on the deposition of glycogen in the gastrocnemius and diaphragm muscles, but the data are not conclusive on this point.

An attempt was made to determine whether growth hormone could affect the glycogen stores of the tissues of the diabetic rat. The considerable variation in severity of the diabetes developed by the rats given alloxan was accompanied by increased variability in the glycogen figures obtained from animals in both fasting and fed states. As shown in Table 4, the car-

diac glycogen tended to be high during fasting, as others have noted in this condition, but the gastrocnemius and diaphragm glycogen values usually were within the normal range. The liver glycogen was greatly elevated in every case, an observation which also has been made previously in alloxan diabetic rats. When glucose was fed to the diabetic rats, no significant storage of glycogen was seen in any tissue. This failure was particularly evident in the diaphragm, for in the normal rats given glucose a massive deposition of glycogen took place in this tissue (from 0.3 per cent in the fasting state to 1.3 per cent after feeding).

On the administration of growth hormone to a series of fasting diabetic rats, the only change which could be detected under these conditions was

TABLE 4. THE EFFECTS OF GROWTH HORMONE ON GLYCOGEN LEVELS IN ALLOXAN-DIABETIC RATS

	No. of animals	Glycogen (mg. per cent)			
		Gastroc. muscle	Heart	Diaphragm	Liver
A. Fasted					
1. Untreated	8	546 ±24	771 ± 57	324 ±18	2460 ±350
2. Growth hormone (1–2 mg. per 100 gm.)	10	574 ±15	963* ± 83	339 ±25	1770 ±270
B. Fed glucose (untreated)	5	584 ±23	908 ±106	401 ±74	3110 ±480

* p (for difference from untreated group) = 0.07.

some tendency toward an increase in the glycogen of the heart (p for increase = 0.035). A few observations, not included in the table, were made on diabetic rats given growth hormone and then fed glucose. Since no large effects were seen, and in view of the wide variation in the amounts of glycogen present in the fed state, no further studies of this type were attempted.

The general lack of parallelism between the effects of growth hormone and of insulin given in either the fasting or fed states makes it seem unlikely that all of the effects of growth hormone were due to increased secretion of insulin. The fact that the smaller doses of growth hormone were not noticeably hypoglycemic (maximum fall about 5 mg. per cent) is in accord with this view, as is also the indication of growth hormone action on cardiac glycogen in the diabetic rats. It is possible that in the diaphragm increased insulin secretion is required for deposition of glycogen, for in normal rats this tissue was sensitive to administered insulin, and the diabetic rat failed to deposit glycogen here after glucose was fed.

DISCUSSION

It has been demonstrated that highly purified growth hormone possesses glycostatic activity in fasting hypophysectomized rats, and that this property may be observed with respect to cardiac and diaphragm glycogen as well as to that of the gastrocnemius. Normal rats given growth hormone, whether fasted or fed carbohydrate, also showed increased glycogen storage in the muscle tissues examined, and in the fasting animals the liver glycogen too was elevated. It is possible that the term "glycostatic," originally applied to the action seen in fasting hypophysectomized animals, might for convenience be broadened to include augmentation of glycogen storage in the fed state, but implications as to the identity of mechanism should be reserved.

Along with the maintenance or increased storage of glycogen in animals treated with pituitary extract or growth hormone, the R.Q. is depressed (Meyer *et al.*, 1937; Russell, 1938b; Russell, 1938c; Greaves *et al.*, 1940; Milman and Russell, 1950). It seems unlikely that these changes could result from an increase in the rate of gluconeogenesis, since they are best demonstrated in animals fed a plethora of carbohydrate and since the animals usually exhibit increased nitrogen retention. These actions could, however, reflect reduction either in the oxidation of carbohydrate or in the formation of fat. There is no experimental evidence as yet to distinguish between effects on these two pathways of carbohydrate utilization. Were the action of growth hormone in increasing glycogen storage a consequence of diminished carbohydrate oxidation or fat formation, then the increase in stored glycogen might come about either because less of the available glucose was being utilized by other pathways; or because preformed glycogen (or glycogen formed at normal rates) was not further metabolized as rapidly as usual.

The observed effects of growth hormone in increasing glycogen storage appear to be at variance with observations made in the isolated diaphragm *in vitro*. In this tissue, the uptake of glucose from the medium and glycogen formation both were increased after hypophysectomy and diminished by pretreatment of the animals with growth hormone (Krahl and Park, 1948; Park and Krahl, 1949; Park and Duaghaday, 1950). It has been widely supposed that the actions of growth hormone and of insulin in such experiments are upon the hexokinase reaction. In the absence of convincing evidence to this effect, however, it seems equally likely that the hormones may affect the rate of supply of energy (presumably as ATP) for the phosphorylation of glucose, rather than the rate of the hexokinase reaction itself. This would imply an effect of the hormones on the oxidation of available substrate, in this instance the glucose of the medium. The data

of Villee and Hastings (1949) indicating increased oxidation of C^{14}-labeled glucose by the diaphragms of hypophysectomized rats, is consistent with this view. It is possible that growth hormone *in vivo* may affect the rate of oxidative generation of phosphate bond energy and through this the rate of glycogenesis as well, but that the catabolism of carbohydrate is inhibited to a greater extent than the rate of glycogen formation. In none of the *in vitro* experiments reported have the conditions been suited to the demonstration of effects of the growth hormone, if there are such, on the further metabolism of preformed glycogen.

The synergism demonstrated here between growth and adrenal hormones is not a new observation. A similar relationship was seen in rats fed glucose after treatment with crude anterior pituitary extracts (Russell, 1940), and more recently a permissive role of cortical hormone in the action of pituitary extracts on glucose uptake by the isolated diaphragm has been suggested (Park and Krahl, 1949). On the other hand, anterior lobe extracts rich in growth hormone will maintain the fasting glycogen levels in adrenalectomized-hypophysectomized rats (Bennett and Perkins, 1945). Whether there is a qualitative or a quantitative difference in the action of pituitary factors in the fed and fasting states is not known. It is possible that the action of cortical extract is somewhat non-specific, dependent on the maintenance of the functional integrity of the tissues so that they are capable of rapid or maximal responses. Or, the growth and cortical hormones may intervene at different levels of carbohydrate utilization; for instance, growth hormone might tend to inhibit the further utilization of glycogen by any route, (i.e., to slow the first stages of oxidation), while the cortical hormone could be affecting more specifically the utilization of carbohydrate fragments for fat formation (see Welt and Wilhelmi, 1950). In this case, the growth hormone alone might be capable of maintaining fasting carbohydrate levels, but for alteration in the disposition of carbohydrate given in quantity, where fat formation is a major route of metabolism, the concurrent action of the two hormones would be required.

An additional point of possible importance which was demonstrated in the present experiments was a differential effect of growth hormone and of insulin on the glycogen of the diaphragm, heart, and skeletal muscle. The diaphragm appeared particularly sensitive to the action of insulin, a small amount of the hormone bringing about a marked increase in the glycogen content of this tissue in the fasting animal. The feeding of glucose to the normal fasting rat resulted in a five-fold accumulation of glycogen in the diaphragm and this was further augmented by pretreatment with insulin. In diabetic rats, glycogen levels in the diaphragm were normal in fasting but were increased scarcely at all by glucose feeding; so that the presence

(or increased secretion) of insulin seems required for large scale glycogenesis in this tissue. Although growth hormone effected increased glycogen deposition in the diaphragm, it was less active here than was insulin. In the heart, on the other hand, where the dose of insulin given had no detectable action, growth hormone was quite effective in increasing glycogen storage, both in the fasted and fed states. The cardiac glycogen tended to be high in diabetic rats, and to increase further on the administration of growth hormone. The glycogen of skeletal muscle was affected in most instances like that of the heart, except that the changes were generally smaller in magnitude. These differences in behavior between the diaphragm and the heart and other muscles suggest that *in vitro* studies of growth hormone action might profitably be extended from the diaphragm, the only muscle examined to date, to heart slices.

SUMMARY

Purified growth hormone given 6 hours before sacrifice to fasting normal rats increased the amount of glycogen found in the gastrocnemius muscle, heart, diaphragm, and liver. In hypophysectomized rats fasted 24 hours, the glycogen of the heart and diaphragm as well as of the gastrocnemius muscle was depleted, and treatment with growth hormone during the fast tended to restore normal maintenance of glycogen stores in these tissues.

Normal rats fasted and then given glucose two hours after growth hormone treatment also exhibited increased storage of glycogen in heart, diaphragm, and gastrocnemius. The minimum effective dose of growth hormone here was about 100 μg. per 100 gm. The presence of small amounts of adrenocortical hormone, but not of the gland itself, was required for the action of the growth hormone in increasing the deposition of glycogen in these tissues in the animals fed carbohydrate.

Insulin, 0.1 unit per kg., increased greatly the amount of glycogen found in the diaphragms of fasting or glucose fed normal rats, but did not affect the other tissues examined. The action of growth hormone on the deposition of glycogen in the heart after glucose feeding was not affected by insulin given simultaneously. In alloxan-diabetic rats, in which normal or elevated muscle glycogen levels were seen in fasting, the glycogen of the tissues (notably that of the diaphragm) failed to rise after the feeding of glucose. A possible increase in cardiac glycogen was indicated when growth hormone was given to fasting diabetic animals. Thus, of the muscles in which glycogen storage was examined, the glycogen of the diaphragm was the most sensitive to the action of insulin, while that of the heart was affected to the greatest extent by growth hormone. Hepatic glycogen levels were altered little by hormone treatment in these conditions.

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INTRAVENOUS ASSAY OF PREPARATIONS CAUSING OVARIAN HYPEREMIA IN THE IMMATURE RAT

S. H. STURGIS,[1] AND V. C. POLITOU

From the Vincent Memorial Hospital and Laboratory, The Gynecologic Service of the Massachusetts General Hospital

IN A PREVIOUS communication (Sturgis and Haour, In Press), the results of a one hour intravenous test for pregnancy were reported. The induction of a bright red ovarian hyperemia in the immature rat by 2 cc. of unaltered urine introduced into the jugular vein was obtained in 96 out of 103 normal pregnancies. It was emphasized that besides the rapidity of the test, this method has the further advantage that the technician is assured through direct vision that all of the prepared material to be tested is added to the animal's circulation. Variations in rate of absorption as encountered with subcutaneous or intraperitoneal injections are thus eliminated. This modification of the widely used rat hyperemia response is particularly appropriate to studies of effective doses of various hormone preparations. Standardization of the dose biologically assayed by each animal is achieved, and small groups of animals may be used to establish response to test solutions.

In this report is recorded our experience with the induction of ovarian hyperemia after intravenous injection of chorionic and pituitary gonadotropins and estrone in an attempt to clarify further the mechanism of this reaction.

MATERIALS AND METHOD

Immature female rats of an original Wistar strain 21 to 24 days old and weighing 30 to 40 gms. are anaesthetized with ether and the external jugular vein gently exposed above the clavicle through a small vertical incision. Aqueous test solutions are made up fresh to insure against loss of potency. The calculated dose in 2 cc. of distilled water is injected slowly with a #27 gauge needle into the vein. The skin is closed with a skin clip. The whole procedure takes about 6 minutes in skilled hands. One hour after the animal has recovered from anaesthesia sufficiently to move about freely, it is sacrificed by 2 minutes of illuminating gas. The ovaries are immediately exposed without blood loss through two flank incisions with a minimum of traction or handling. A positive reaction is one in which one or both ovaries are a deep cherry red, a color value as intense as the spleen, but of a different hue. An equivocal reaction is read when one or both ovaries show more hyperemia than the adjacent uterine horn, but not as intense

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[1] Present address: Peter Bent Brigham Hospital, Boston, Mass.

as liver or spleen. In a negative reaction, both ovaries are pearly white, with less color than that of the uterus.

RESULTS

1. *Chorionic Gonadotropin*

Chorionic gonadotropin in tablet form, assayed to contain 10 International units per mg.[2] was weighed and dissolved in distilled water directly before injection. Serial dilutions were prepared to contain known amounts in 2 cc. The Minimal Effective Dose (MED)[3] of this hormone in our strain of rats was 2.5 I.U. (See Table 1.)

TABLE 1. HYPEREMIA RESPONSE TO CHORIONIC GONADOTROPIN 1 HOUR AFTER INTRAVENOUS INJECTION

Dose I.U.	Number of animals	Positive	Equivocal	Negative
0.000 (distilled H_2O)	15	0	5	10
0.156	6	0	3	3
0.312	4	0	2	2
0.625	4	1	2	1
1.250	10	2	8	0
2.500	10	7	3	0
5.000	10	9	1	0
10.00	30	25	5	0

It will be seen in Table 1 that a third of the controls injected with distilled water showed an equivocal pink reaction in one or both ovaries. None of them, however, were positive. In reading pregnancy tests, all equivocal reactions are regarded as negative, and in the tables to follow, only the bright red positive responses are recorded. Since the sensitivity of the ovarian color change has been shown to vary as a function of the time between injection and examination (Zondek and Sulman, 1947), it seemed possible that smaller doses than 2.5 I.U. might produce consistently positive hyperemia if more than 1 hour elapsed after intravenous injection. It was of interest as well to study the duration of a positive response after the larger doses had been injected.

Table 2 shows the response obtained at intervals of 1, 3, 6, 8 and 18 hours after different doses of intravenous chorionic gonadotropin. It is to be noted that the response to the MED of 2.5 I.U. at one hour was sustained at 3 hours, then faded off. Smaller doses did not cause more hyperemia after longer intervals of time. It took the relatively huge dose of 20 I.U. or more per rat to maintain a positive hyperemia in 3 out of 4 animals 8 and 18 hours after intravenous injection. It seems clear, then, that the ovarian

[2] Supplied through the courtesy of E. F. Cook, M.D., U. S. Pharmacopeia, Phila., Penna.

[3] Minimal Effective Dose defined as the smallest amount necessary to produce a positive reaction in one or both ovaries in 70% of animals used.

response to the hyperemia factor in this hormone is most sensitive at from one to three hours after intravenous injection. Such a prompt response suggests a direct action on the ovarian vascular tree. Variations of sensitivity over longer intervals of time after subcutaneous or intraperitoneal injections may be due to complex inter-relations of absorption and excretion rates.

2. *Luteinizing Hormone*

A number of investigators, using subcutaneous or intraperitoneal injections, have ascribed the hyperemia response to the LH component of gona-

TABLE 2. EFFECT OF TIME FROM INJECTION TO EXAMINATION ON OVARIAN HYPEREMIA INDUCED BY INTRAVENOUS CHORIONIC GONADOTROPIN

Dose I.U.	1 hour No.—Pos.	3 hours No.—Pos.	6 hours No.—Pos.	8 hours No.—Pos.	18 hours No. —Pos.
0.312	4— 0	4—0	4—0	4—1	4—0
0.625	4— 1	4—1	4—0	4—0	4—0
1.250	10— 2	4—1	4—0	4—0	4—1
2.500	10— 7	4—4	4—0	4—1	3—2
5.000	10— 9	4—3	4—2	4—1	4—1
10.00	30—25	4—3	4—3	4—2	4—3
20.00	10— 9	4—2	4—2	4—4	4—4
40.00	4— 4	4—3	4—0	4—4	4—2
80.00	4— 3	4—3	4—4	4—4	4—4

dotropic preparations (Kupperman *et al.*, 1946; Lloyd *et al.*, 1949; Zondek and Sulman, 1947). Most workers examined their animals 2 to 24 hours after injection. A delayed response after several hours could involve stimulation and release of the animal's own anterior lobe principles. Such a possibility is supported by Fried and Rakoff (1950) who studied the effect of a "pituitary synergist" added to chorionic gonadotropin in normal and hypophysectomized rats. The latter animals, killed 2 hours after intraperitoneal injection, showed no ovarian hyperemia unless doses ten to twenty times the Minimal Effective Dose in normal controls were used. They concluded that "it would therefore appear that chorionic gonadotropin requires the cooperation of an anterior lobe factor, presumably FSH, in order to produce the ovarian hyperemia effect." This is in accord with the statement of Zondek *et al.* (1947) that "the hyperemia reaction is elicited by prolan B only if prolan A is present." To check these reports, we found the MED for LH #234 in our intact animals was 4 gamma. If, as suggested above, the hyperemia response requires the presence of FSH, one would expect negative results with pure LH after oblation of the pituitary. In our hypophysectomized animals there were 5 out of 10 positive reactions to 4 gamma and 3 out of 10 positive to twice this dose (Table 3). The

TABLE 3. RESPONSE OF INTACT AND HYPOPHYSECTOMIZED IMMATURE RATS ONE HOUR AFTER INTRAVENOUS INJECTION OF PURIFIED LH (FWP#234, ARMOUR)

Dose (mgs.)	Intact No.—Pos.	Hypophysectomized No.—Pos.
0.00 (distilled H_2O)	10—0	
.00025	10—0	10—1
.00050	10—1	10—0
.001	10—0	10—0
.002	10—1	10—2
.004	10—9	10—5
.008	10—8	10—3

hyperemia here elicited, contrary to the results of Fried and Rakoff (1950) with chorionic gonadotropins, was produced in the absence of an augmenting FSH factor from the pituitary. The possibility yet remains that the preparation LH #234 might contain a trace of FSH sufficient as a synergist to account for the hyperemia we obtained in the hypophysectomized group. The investigation of this possibility was beyond the scope of the present study.

Only minute amounts of purified LH are required to induce hyperemia. The intravenous technique offers a practical way of comparing the potency of different preparations. Table 4 records the assay by this method of two other LH extracts.

TABLE 4. POTENCY ASSAY OF LH #7E20 (ARMOUR) AND LH #PCF70 (ARMOUR LH STANDARD) BY OVARIAN HYPEREMIA RESPONSE IN ONE HOUR IN INTACT ANIMALS

Dose (mgs.)	LH #7E20 No.—Pos.	LH #PCF70 No.—Pos.
0.00	5— 0	
0.000125		10—2
0.00025	10— 4	10—7
0.0005	10— 2	10—8
0.001	10— 8	5—4
0.002	10— 6	5—3
0.004	10— 9	5—4
0.008	16—14	

The lowest dose of LH 7E20 that gave 70% or more positives was one gamma, whereas the Armour LH Standard (PCF-70) induced 70% reactions with a one quarter gamma dose. This suggests that the latter preparation is in the neighborhood of 4 times more potent than LH 7E20 and 16 times more potent than LH 234 (Table 3) with regard to the production of ovarian hyperemia in our animals.

3. *Follicle-Stimulating Hormone*

At present even the most highly purified FSH preparation from animal sources probably contains some LH contamination. Urine or pituitary

extracts with a high titer of the follicle stimulating factor do not usually cause ovarian hyperemia (Kupperman *et al.*, 1948), although false positive pregnancy tests in menopausal patients (Farris, 1944) and at mid-cycle (Farris, 1946) have been reported. It may be assumed in these cases that sufficient LH was present to cause the reaction. Riley, Smith and Brown (1948) obtained negative response to 40 U. of an FSH extract, and ascribed positive reactions from 200 U. to traces of LH present in their preparation.

Having established the MED in gamma of a standard LH preparation necessary to induce ovarian hyperemia, we have tested two purified FSH[4] extracts by the same technique in order to estimate their contamination by LH in terms of weight equivalents of the standard. Table 5 gives

TABLE 5. INDUCTION OF OVARIAN HYPEREMIA BY PURIFIED FSH EXTRACTS ONE HOUR AFTER INTRAVENOUS INJECTION IN INTACT IMMATURE RATS

FSH #KD62C (Armour)		FSH #39DS (Armour)	
Dose (mgs.)	No.—Pos.	Dose (mgs.)	No.—Pos.
0.023	10—1	0.150	2—0
0.046	10—5	0.375	10—2
0.093	10—4	0.500	10—7
0.186	10—7	0.750	10—7
0.375	5—4	1.000	5—2

the results. The extract KD 62C induced positive reactions in 7 out of 10 animals in a dose of 186 gamma; one quarter this dose, 46 gamma, still gave 50% positives, while 23 gamma was relatively ineffective. One can conclude that in 186 gamma, there is at least an amount of the hyperemic factor equivalent to the MED of the Armour LH Standard (0.25 gamma), but probably not as much as this in 23 gamma of this FSH. The contamination therefore, is more than 0.13% but less than 1% in equivalent weights of the LH Standard. The results from FSH 39 DS, a purer extract, appear to be more clear cut. Similar calculations indicate from this table a contamination of more than 0.05% but less than 0.06% in terms of LH Standard.

When relatively large doses of these impure protein extracts are given intravenously, they are often poorly tolerated. Injection of 1 mg. of 39 DS had to be extremely slow. The animals did not recover readily after anaesthesia and their poor response (two positive out of five) reflects the toxicity of the amount given.

4. *Estrone Sulphate*

The use of ovarian hyperemia as a diagnostic test for pregnancy is based on the assumption that the reaction is entirely due to increase in urinary

[4] All the purified LH and FSH extracts were supplied through the courtesy of Dr. I. Bunding, Armour & Co., Chicago.

chorionic gonadotropin in early gestation. There is also a progressive increase in excretion of estrogens at this time. D'Amour (1940) observed that estrogen apparently can cause stimulation of the anterior pituitary with discharge of gonadotropin; Fevold, Hisaw and Greep (1936) stated that the first effect of such stimulation is an increased secretion of LH. In clinical reports, Brown and Bradbury (1947) and Lloyd *et al.* (1949) find evidence in support of this view. Furthermore, Farris (1946) has reported the production of an ovarian hyperemia after estrogen injection in immature rats. We have been unable to provoke a significant hyperemia from urine samples obtained after estrogen medication in normal subjects (Sturgis & Haour, In Press). It seemed worthwhile, however, to test immature rats intravenously with varying estrogen doses to evaluate the possibility that the hyperemia caused by pregnancy urine could be due in part to its estrogen content. The

TABLE 6. OVARIAN HYPEREMIA INDUCED BY ESTRONE SULPHATE INTRAVENOUSLY ONE HOUR AFTER INJECTION

Dose (mgs.)	No. animals	No. positive
0.5 –1	15	0
1.25–2.5	14	0
5.0	10	0
10.0	10	5
20.0	10	6

effect of estrogen might be due to a direct dilatation of ovarian capillaries, or to stimulation of the animal's own hypophysis causing a secondary hyperemic response. Estrone sulphate[5] freshly prepared in distilled water, was therefore given in large doses and the animals sacrificed one hour later to test the first hypothesis, and a wider range including smaller doses was tested at 1, 2, 3 and 4 days after intravenous injection to evaluate the second of the above possibilities. In Table 6, it is seen that when huge doses were used, a positive response was evoked in half the rats one hour after injection. Doses of 20 mgs. intravenously were poorly tolerated by the intact animals and it was not possible for this reason to run a parallel series in hypophysectomized material.

Table 7 gives the results from different dilutions of Estrone Sulphate when groups of rats were examined at intervals of 1 to 4 days after intravenous injection. Again, relatively huge doses (5 to 20 mgs./rat) gave only inconstant hyperemia 72 or more hours later. Several explanations are possible. The anterior pituitary of these animals may not have been mature enough to respond to estrogenic stimulation. It may be that, as has been suggested (Brown & Bradbury, 1947), an optimum dose of estrogen causes

[5] Estrone Sulphate generously supplied by Dr. E. C. Reifenstein, Ayerst, McKenna and Harrison.

a sudden, transient discharge of gonadotropin, and the resultant hyperemia may have faded in a few hours as indicated in Table 2, and thus may have been missed. Perhaps estrone sulphate is not a potent estrogenic compound in this regard. Finally, it is possible that even the lowest dose of 1 gamma may have been much too large, so that a depressing rather than a stimulating effect was produced. It is difficult to understand why, at 72 hours but not before, a few animals (6 out of 12) reacted with positive hyperemia after doses of 5 to 20 mgs. It would be necessary to run many more groups of animals at closer intervals of time with a wider dose range of other estrogenic substances to settle whether or not estrogen under optimum conditions can produce a discharge of autogenous pituitary hormone containing the hyperemic factor. From the above results, however, it seems unlikely that the amount of estrone in 2cc. of an unaltered pregnancy urine when given intravenously could possibly be wholly responsible for the typical positive reaction obtained in one hour (Sturgis & Haour, In Press). It seems probable as well that estrone is not involved in a hyperemia reaction to pregnancy urine obtained 24 hours after subcutaneous injection. We have not, however, tested the possibility of a synergistic action of estrogen and chorionic gonadotropin on production of hyperemia.

TABLE 7. EFFECT OF ESTRONE SULPHATE IN PRODUCING OVARIAN HYPEREMIA ONE TO FOUR DAYS AFTER INTRAVENOUS INJECTION

Dose (mg.)	24 hours No.—Pos.	48 hours No.—Pos.	72 hours No.—Pos.	96 hours No.—Pos.
0.001	5—0	5—0	5—1	5—0
0.01	5—0	5—1	5—1	5—0
1.00	5—0	5—0	5—0	5—1
5.00	5—0	5—0	6—2	5—2
20.00	5—2	5—0	6—4	5—2

SUMMARY

The maximum sensitivity of the hyperemia response in the rat ovary to chorionic gonadotropin given intravenously was found at one to three hours after injection.

The potency of the hyperemic factor in three purified LH pituitary extracts was tested. As little as one quarter gamma of a standard LH preparation was found effective one hour after intravenous injection in immature animals. Since hypophysectomized rats also responded, it is probable that this is a direct action on the gonad in which the animal's own pituitary plays little or no part.

The hyperemia induced by relatively large amounts of two purified FSH extracts is considered due to their contamination by the hyperemic factor associated with preparations of LH. A comparison of the per cent of such

contamination was made on the basis of effective dosage of the LH Standard previously established.

Estrone sulphate gave negative results under the conditions described. This does not preclude the possibility that under other conditions estrogens might stimulate production of the hyperemic factor from the animal's own hypophysis.

The advantage of using the intravenous techique is the standardization of the amount of test extract added under vision to the circulation. The disadvantage of using a subjective endpoint is considerably offset by regarding as negative all color responses less marked than an unequivocal bright cherry-red.

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RADIOCALCIUM AS AN INDICATOR IN THE STUDY OF THE ACTION OF ESTRADIOL ON CALCIUM METABOLISM

J. GOVAERTS, M. J. DALLEMAGNE AND J. MELON

From the Institut de Thérapeutique Expérimentale, Institut de Physique Nucléaire and Institut de Minéralogie et de Cristallographie, University of Liége, Liége, Belgium

IT HAS been known, since the observation of Kyes and Potter (1934), that during ovarian follicular activity the endosteal cavity of long bones of female pigeons and other birds becomes filled with abundant bony trabeculae. Landauer *et al.* (1938) reported that it is possible to produce this type of new bone formation in pigeons by estrogen (folliculin) injections causing the bone marrow to be resorbed and replaced by newly formed organic trabeculae which later become calcified. Riddle and Reinhart (1926) ascertained that the blood calcium content of female pigeons undergoes a marked elevation in the period just preceding egg-shell formation and that the hypercalcemia is maintained until shell formation is completed. Riddle and Dotti (1936) produced similar effects on the blood calcium content of normal and of castrated or hypophysectomized pigeons and doves by the administration of estrogenic hormones. Clavert and Benoit (1942) have also found that the calcium content of the plasma rises and have noted further that the absorption of calcium from the gastrointestinal tract is increased during normal and experimental hyperfolliculinemia. When the estrogen injections are discontinued the endosteal bone regresses (Clavert, 1948) and the calcium excretion is greatly increased. Estrogen injections produce the same effects in male pigeons during testicular activity (Bloom, McLean and Bloom, 1942). Dallemagne and Melon (1947) have shown that the new medullary bone, like old bone, is principally formed of tricalcium phosphate.

During the past few years, radiophosphorus has been extensively used for the study of endosteal bone formation (Hevesy and Hahn, 1937; Chaikoff, 1943; O'Neil *et al.*, 1948). Using this radioisotope (Dallemagne *et al.*, 1947; Govaerts and Dallemagne, 1948), we have shown the phosphorus exchange of several tissues of pigeons is significantly accelerated by estrogen administration and that the phosphorus of the newly formed bone is provided by the original skeleton. Radiocalcium has also been used in the study of bone metabolism by different investigator (Pecher, 1941; Armstrong and Barnum, 1948; Copp *et al.*, 1947). Campbell and Greenberg

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(1940) have shown that 23.6% of this radioisotope given by stomach tube to the rat appears in the blood and that 21.1% of the dose may be recovered in the skeleton sixty-nine hours after its administration.

In our opinion, radiocalcium seems to be more suitable than radiophosphorus in the study of bone because calcium, unlike phosphorus, is almost entirely situated in bone. For example, Campbell and Greenberg found the ratio $\frac{Ca^{45}\ \text{bones}}{Ca^{45}\ \text{muscles}}$ to be 0.667 while in the case of radiophosphorus the corresponding ratio was 0.023. It is thus apparent that a study of the influence of estrogen on bone metabolism by means of radiocalcuim (Ca^{45}) should be of interest.

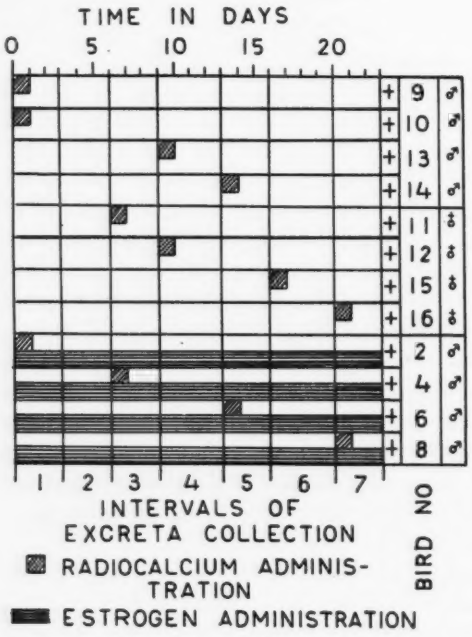

FIG. 1. Schedule of the experiments indicating the time of administration of radiocalcium and estrogen to pigeons and the periods of collection of the excreta.

MATERIAL AND METHODS

Twelve pigeons each weighing about 425 gm. were used in these experiments. In order to determine the calcium excretion under normal conditions, all the animals were placed in metabolism cages for a period of nine days before administration of estrogen of radiocalcium. Excreta were collected over 3–4 day intervals during the preliminary

and the experimental periods. The schedule of administration of radiocalcium and estrogen is indicated in Figure 1. All the birds were sacrificed on the same day, but the injections of the radioisotope were scheduled in such a manner that the animals were sacrificed at intervals of 3 to 23 days after the administration by subcutaneous injection of a single dose of 2.4 or 4.8 mg. calcium tagged with radiocalcium.*

The following three groups of pigeons were studied (see Fig. 1):

a) Control birds (numbers 9, 10, 13 and 14): four male birds, no estrogen,

b) Female birds (numbers 11, 12, 15 and 16) in normal ovarian activity,

c) Male birds (numbers 2, 4, 6 and 8) in normal seasonal testicular activity, given daily 0.25 mg. alpha estradiol dipropionate (diovocyclin, Ciba) by intramuscular injection during twenty-three days.

At the end of each of the numbered time intervals (Fig. 1) the excreta were collected, dried, powdered and ashed in an electric furnace at 600° C. The ash was dissolved in hydrochloric acid and suitable aliquots were pipetted into dishes and evaporated to dryness for measurement of the radioactivity.

When each pigeon was sacrificed a blood sample was obtained and the digestive tract and its contents were removed. These materials were either wet or dry ashed and the ash dissolved in hydrochloric acid. The following bones were also removed: breastbone, tibia, femur, humerus, radius and ulna. The bones were first cleaned and then treated with carbon tetrachloride to remove lipids. They were cut longitudinally in two parts, dried at 105° C. and weighed before calcination at 600° C. The ash was dissolved in hydrochloric acid. Suitable aliquots were taken from the solution of each sample for chemical and radio-activity measurements.

Calcium was determined according to a standard analytical procedure (Clark and Collip, 1925). The radio-activity measurements were made with a thin window cylindrical Geiger-Muller tube and all measurements were corrected for the resolving time losses and for self-absorption by the sample. All activities were compared to that of a standard sample prepared in the same manner and counted in the same geometrical conditions as the samples.

RESULTS

a) *Calcium excretion.* The quantity of total calcium excreted during the 23 days of the experiment by the female birds in normal ovarian activity is similar to that excreted by the male controls during the same period as shown from the data reported in Table 1. As a result of estrogen administration the total calcium excreted by the male birds during 23 days is considerably reduced (Table 1). The calcium content of the excreta expressed as percentages of the ash is decreased by the estrogen injections (upper part of Fig. 2) and the lowest excretion rate is observed after seventeen days of treatment.

Greater variations are observed as to the excretion of the total calcium by the control animals than is seen in the same aninals for the injected radiocalcium (Table 1). This finding is due to the fact that total calcium

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* The radiocalcium used in this study was obtained from the Oak Ridge National Laboratories under an allocation of the U. S. Atomic Energy Commission.

TABLE 1. DATA AS TO THE EXCRETION OF TOTAL CALCIUM AND RADIOCALCIUM BY BIRDS AND THE CONCENTRATION OF Ca^{45} IN THEIR TISSUES

	Control males				Females in normal ovarian activity				Estrogen treated male animals			
Time in days after administration of radiocalcium	10	14	23	23	3	7	10	13	3	11	17	23
Bird number	14	13	10	9	16	15	12	11	8	6	4	2
Injected dose mg. Ca	2.40	2.40	4.80	4.80	2.40	2.40	4.80	4.80	2.40	2.40	4.80	4.80
Injected Ca found in the excreta (mg.)*	0.95	1.39	3.32	2.29	0.51	0.95	1.36	1.88	0.46	0.39	0.55	1.95
Per cent of the dose excreted	39.85	57.88	69.19	47.75	21.20	39.47	28.29	39.16	9.60	8.08	11.38	40.71
Total Ca excreted in 23 days (mg.)	136.45	364.63	789.05	640.20	62.70	123.52	238.30	343.71	81.36	47.19	143.65	233.51
Digestive tract and contents (specific activity $\times 10^3$)	51.2	17.7	3.7	19.7	10.00	4.98	1.93	2.82	71.5	27.5	11.9	15.9
Blood specific activity ($\times 10^3$)	35.5	25.1	9.7	18.7	11.61	5.80	4.87	2.92	186.0	109.4	49.0	25.9
Total Ca in blood mg. %	5.89	7.73	5.01	6.85	7.75	7.17	7.72	7.67	24.98	25.13	20.87	15.72

* Calculated by multiplying the proportion of Ca in mg. to counts in the injected material by the number of counts in the excreta.

excretion depends in large part on the quantity of food calcium absorbed from the gastrointestinal tract, a factor which is probably rather variable.

The period-to-period excretion of labeled calcium by the male birds receiving daily injections of estrogen is reported in the lower part of Figure 2. The excretion of the injected raidocalcium is greater during the first

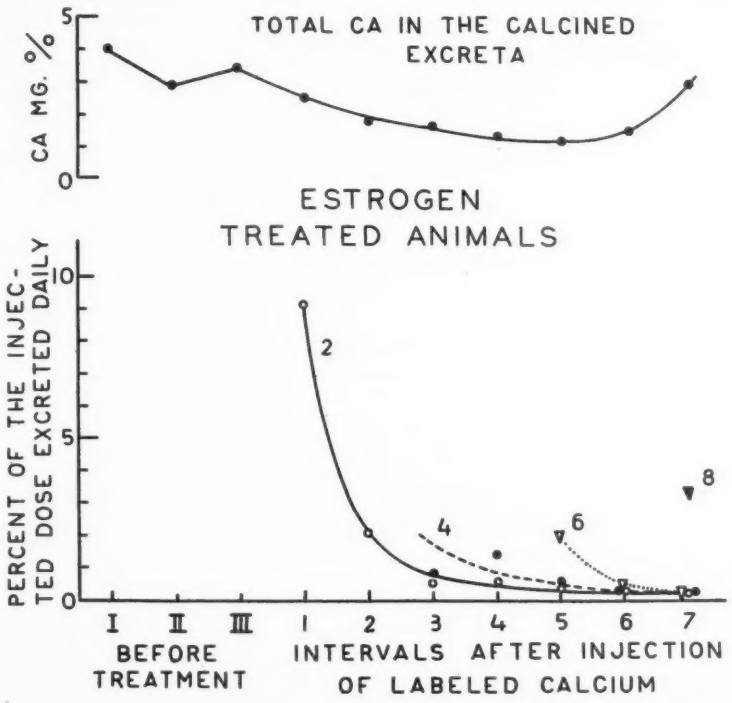

FIG. 2. *Upper Curve.* The mean total calcium content of the calcined excreta of the estrogen treated birds. *Lower Curves.* The mean quantity of radiocalcium excreted daily by the same birds.

days[1] following its administration than during subsequent days. A comparison of the curve for the estrogen treated bird (No. 2 in Fig. 2) and the curve for the control males (Fig. 3) indicates that the radiocalcium administered at the beginning of estrogen injections is excreted in the same quantity as in the control birds. If the radiocalcium is injected later during estrogen treatment much more of the injected dose is fixed by the bird (Fig. 2).

[1] The values of "Per cent of Administered Dose Excreted Daily" plotted on the ordinates of Figures 2 and 3 are mean values and were obtained by dividing the result found with the excreta collected during each period by the number of days in the period.

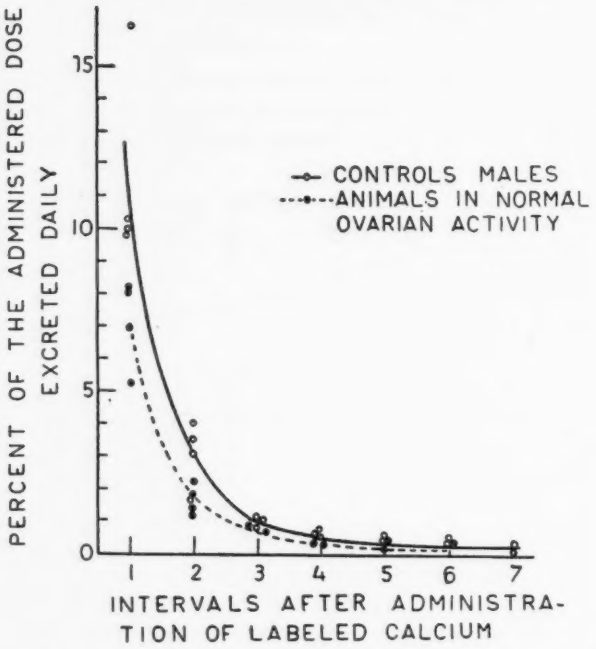

FIG. 3. The quantity of radiocalcium excreted daily by the control male birds and by the females in normal ovarian activity.

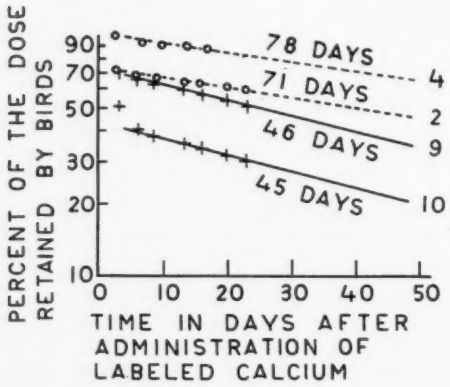

FIG. 4. Variations with time of the percentage of administered radiocalcium retained by the estrogen treated and control birds showing the biological half-life of calcium in each.

Maximum fixation occurs when the estrogen treatment is started about 7 days before the administration of the radiocalcium.

Figure 3 depicts the percentage of the injected dose of radiocalcium excreted daily[1] by the birds in normal ovarian activity compared with that of the control birds. In the case of the former animals the radiocalcium excretion seems to be a little lower than of the control birds.

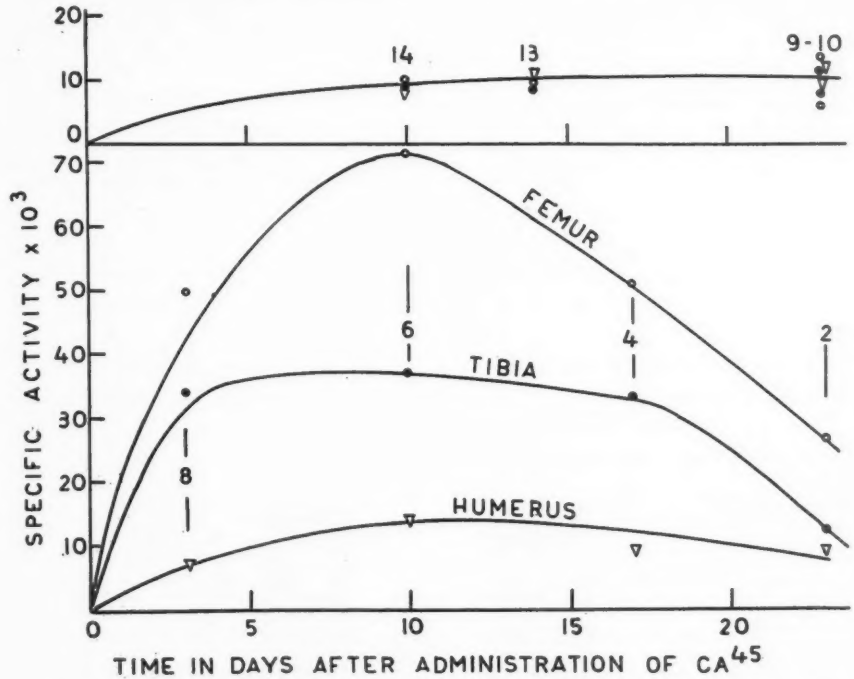

FIG. 5. Variations with time after administration of radiocalcium in the specific activity of bones of the control animals (upper curve) in which no differences were found among several bones and (lower curves) of the estrogen treated animals.

The half period of retention in the body can be estimated from a plot of the data on semi-logarithmic paper in which the percentages of the injected dose remaining in the animals are plotted on the logarithmic axis and time on the regular axis. As shown in Figure 4, curves analogous to a radioactive decay curve are obtained. A biological half-life of calcium of 45–46 days is found for the control birds (Nos. 9 and 10). A very much higher value of 71–78 days is obtained for the folliculin treated animals (Nos. 2 and 4). With female birds in normal ovarian activity the biological half-life of calcium is about 60–67 days.

b) *Calcium of the soft tissues.* The observations with estrogen treated birds (Nos. 2, 4, 6 and 8) present the interesting fact of a very high specific activity of the blood (Table 1). However, the specific activity of the digestive tract and contents is nearly the same in the estrogen treated as in the control animals. The specific activity of the digestive tract and contents is very low in birds in normal ovarian activity.

c) *Calcium in the bones.* In the control animals, the tracer uptake is low and of a uniform amount in all bones studied as is shown in the upper part of Figure 5. As shown in the lower part of the same figure the femur and tibia are noteworthy because of a particularly large uptake of the tracer after estrogen injections. The uptake of radiocalcium in the ulna and radius of the estrogen treated animals was also found to be higher than in the controls but no significant difference was observed between control and experimental birds with other bones such as the humerus and breastbone. The same interpretation with regard to the skeleton can be made for the birds undergoing normal ovarian activity but the quantity of radiocalcium fixed in the bones of these birds is not as high as in the case of the estrogen treated animals.

Bone radioautographs were prepared from the different longitudinally cut bones by placing them on a photographic film. A typical result obtained is present in Figure 6 showing radiocalcium deposition in the bone epiphyses and diaphyses as well as in the endosteal bone.

FIG. 6. Radioautographs of radii of an estrogen treated bird (No. 8), a male control (No. 10), and bird No. 11 in normal ovarian activity.

DISCUSSION

The results show that estrogen influences greatly calcium metabolism not only in the skeleton but also in the soft tissues. The uptake and retention of the injected calcium are both largely increased by daily injections of 0.25 mg. alpha estradiol dipropionate. The highest degree of retention is observed when the labeled calcium is administrated about seven days after estrogen injections are started. The rate of radiocalcium excretion is slowed by estrogen administration and the half-life of calcium retention in the body is about 71–78 days instead of 45–46 days as found with the control birds. To a smaller degree the same conclusions can be drawn from the results obtained with the birds in normal follicular activity.[2] In the latter, the half-life of calcium in the body is about 64 days.

Not only the total calcium concentration but also the amount of radiocalcium in the blood are very high in the estrogen treated animals. However, in the same animals the quantity of radiocalcium in the digestive tract and its contents parallels that of the controls. In contrast to the estrogen treated male birds, the female animals with normal sexual cycles show a normal calcium level in the blood and lower amount of radiocalcium in the digestive tract and contents. These data show that exogenously administered estrogen in the dose given increases the radiocalcium retention much more than the hormone physiologically secreted by the ovaries.

Radiocalcium, as is demonstrated by the radioautographs, was present not only in the endosteal bone but also in the diaphyses of the femora and tibiae. Radiocalcium was also present in the diaphyses of the other bones, but in a very much lower amount. The rapid exchange of calcium in the femur and tibia, in contrast to that of other bones, seems to be in contradiction to the hypothesis of a physiological unity of the skeletal system (Roche, 1939).

Nearly the same specific activities are found in the humerus and breastbone in estrogen treated animals as in the controls. Although calcium and phosphorus concern mainly the same molecule, tricalciumphosphate, the estrogen action on calcium metabolism in these bones seems to be very weak compared to our previous results obtained with radiophosphorus.

SUMMARY

The influence of estrogen on calcium metabolism in soft tissues and skeleton has been studied in pigeons by the use of radiocalcium. Daily estrogen injections produce an increase of calcium metabolism to a higher degree than can be observed to occur by normal hormonal secretion. The excre-

[2] That ovarian activity occurred is proved by the volume of ovaries and the presence in them of many follicles.

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tion of calcium is decreased by estrogen injections and at the same time the over-all retention of calcium is increased, yielding a biological half-life of calcium of about 75 days instead of 45 days in the controls. The distribution of calcium in bone was also studied by the radioautographic technic. The new medullary bone of the femur and tibia is very rich in injected radiocalcium. A comparison is made between calcium and phosphorus in bone metabolism.

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OBSERVATION ON THE *IN VIVO* INACTIVATION OF TESTOSTERONE PROPIONATE BY THE LIVER OF THE WHITE RAT[1]

JOHN T. GRAYHACK AND WILLIAM WALLACE SCOTT

From the James Buchanan Brady Urological Institute, Johns Hopkins Hospital, Baltimore

THE mechanism by which the liver inactivates[2] various steroid hormones has been the subject of numerous investigations since Zondek (1934) first demonstrated the inactivation of estrogen by this organ. Interest in the metabolism of estrogens seems to have been more extensive than interest in the metabolism of androgens. Six years after Zondek's observation with estrogens, Biskind and Mark (1939) demonstrated that pellets of testosterone propionate implanted in the spleen did not effect weight changes in the prostate or seminal vesicles of castrate rats. They observed that pellets implanted subcutaneously, or into a subcutaneously transplanted spleen, caused a marked increase in the size of the male accessories. From this evidence, they suggested that the liver was the site of inactivation of testosterone propionate. Later, these observations were extended to methyl testosterone and testosterone (Burrill and Greene, 1942). The former caused slight prostatic enlargement when implanted in the spleen. Burrill and Greene (1940) showed that endogenous testicular androgen was ineffective when the testis was transplated to sites drained by the portal vein.

Biskind and Biskind (1943) reported that animals deficient in "Vitamin B complex" continued to inactivate splenic androgen although they were unable to inactivate splenic estrogen. Selye's (1941) observations on the anesthetic effect of various steroids in partially hepatectomized rats also seemed to suggest that the method by which the liver rids the body of androgen was less liable to depletion than that by which it rids the body of estrogen. Jailer (1948, 1949) has shown that inanition and, more specifically, protein deficiency decrease the ability of the rat's liver to inactivate estrogen. Shipley and György (1944) obtained similar results in rats with

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[2] Throughout the article the word "inactivate" is used to indicate prevention of the normal physiological function of a substance on the body. The prevention of this action may be the result of a metabolic alteration, a simple elimination before reaching sensitive end organs, or a combination of these.

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experimental dietary cirrhosis; Pincus and Martin (1940) demonstrated that sublethal doses of carbon tetrachloride increased the effectiveness of administered estrogen in the rat.

We have been interested in determining the extent of the ability of the intact animal to inactivate androgen and the mechanism by which this is accomplished. In these studies, we have implanted pellets of crystalline testosterone propionate in the spleens of castrate rats subjected to various stresses thought to affect liver metabolism.

Groups of castrate male albino rats of the Wistar strain with intrasplenic pellets of testosterone propionate[3] were subjected to carbon tetrachloride poisoning, to complete inanition, to moderate inanition, and to a dietary intake of glucose and tap water only. Under similar conditions, male rats of the Sprague-Dawley strain were subjected to complete inanition and hypophysectomy.

EXPERIMENTAL

1. *Carbon tetrachloride poisoning.* In this group, a total of 44 adult male rats of the Wistar strain, weighing about 300 gm., were used. Of these, 33 were castrated and, after a period of 5 weeks, pellets of crystalline testosterone propionate (8.5–13.5 mg. each) were implanted either into the spleen or beneath the skin. Three days after implantation, rats of Groups D, G, and H were begun on subcutaneous injections of carbon tetrachloride every other day according to the following schedule: Injection (1) 0.1 cc.; (2) 0.2 cc.; (3) 0.2 cc.; (4) 0.25 cc.; (5) 0.25 cc.; (6) 0.30 cc.; (7) 0.4 cc.; (8) 0.50 cc.; (9) 0.50 cc.; (10) 0.50 cc.; (11) 0.50 cc. The day after the 8th injection, abdominal exploration in a few rats revealed yellow, friable, damaged liver. Two days after the final injections, the rats were sacrificed. Adrenal, pituitary and prostatic weights were obtained to the nearest 0.5 mg. on a torsion balance. The pellets were removed, cleaned, dried and weighed to the nearest 0.1 mg. on a chain balance. Hematoxylin and eosin sections of the liver and kidney were made from representative animals of each group.

During the entire procedure, all rats were offered 20 gm. of Purina Laboratory Chow daily. Body weights were obtained at least once weekly and at autopsy.

Results. The results for this group of animals are shown in Table 1. It will be noted that loss in body weight of the animals receiving carbon tetrachloride is slight (Groups B, D, G, and H). In castrate animals with subcutaneous testosterone pellets (Groups F and H), the total prostatic weights were found to exceed that of the normal controls (Group A) at the termination of the experiment. This indicates that the amount of androgenic activity available to the organism from these pellets exceeded that normally supplied by the testis. Prostatic weights in animals with intrasplenic testosterone propionate pellets (Groups E and G) were comparable to those in the castrate rats (Groups C and D). This is true of the groups receiving carbon tetrachloride (G) as well as those receiving none (E).

[3] Pellets of testosterone propionate were kindly supplied by Dr. E. C. Reifenstein of Ayerst, McKenna and Harrison, Ltd.

TABLE 1. RESULTS OF CARBON TETRACHLORIDE STUDY

Group	Carbon tetrachloride injections	Subcutaneous testosterone propionate implantation	Intrasplenic testosterone propionate implantation	Number of animals	Initial body weight, grams	Final body weight, grams	Average weight change, grams	Total prostate weight, mg.	Ventral prostate weight, mg.	Posterior prostate weight, mg.	Milligrams testosterone propionate per rat per day
Stock diet Intact											
A				5	300	315	15	550	368	182	—
B	Yes			6	315	298	−17	390	273	117	—
Stock diet Castrate											
C				4	330	340	10	58	27	30	—
D	Yes			7	305	284	−21	66	40	26	—
E			Yes	6	303	316	13	67	37	30	0.12
F		Yes		3	335	362	27	1138	784	354	0.13
G	Yes		Yes	9	331	298	−33	70	40	30	0.13
H	Yes	Yes		4	314	309	− 5	1138	751	387	0.14

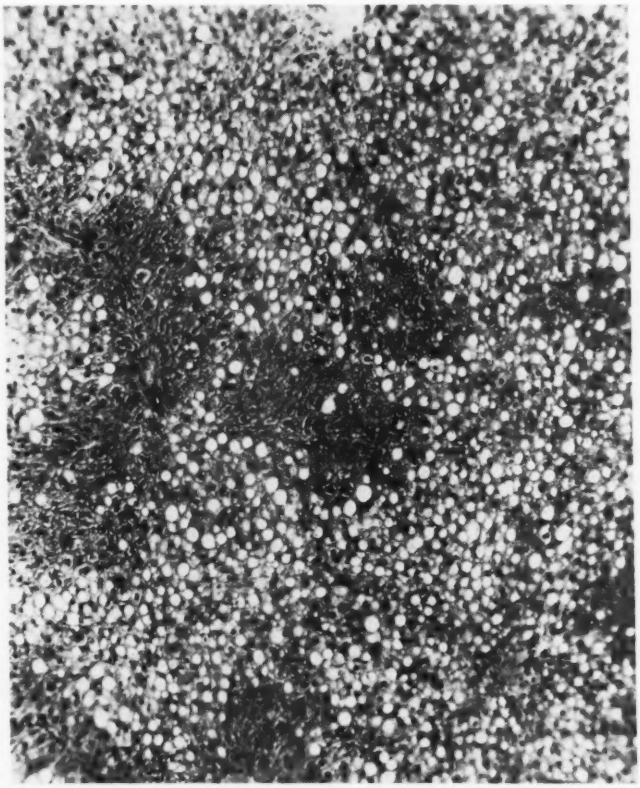

FIG. 1. Photomicrograph of liver of rat receiving carbon tetrachloride according to schedule described in text. (×100)

Figure 1 is indicative of the liver damage present at the termination of the experiment in those rats receiving carbon tetrachloride. Dr. Morgan Berthrong of the Department of Pathology estimated that at least 50% of the functioning liver parenchyma was severely damaged by this toxin. Despite this, the liver was capable of preventing at least physiological amounts of androgenic material implanted in the spleen from reaching the general circulation and causing prostatic growth.

2. *Starvation.* In testing the effect of complete starvation on the inactivation of splenic androgen, rats of both the Wistar and Sprague-Dawley strains were used. Thirty-nine 175 gm. rats of the Wistar strain were castrated. Three and one-half weeks later, during which time the animals received Purina Laboratory Chow ad libitum, pellets of crystalline testosterone propionate (10.0–13.5 mg.) were implanted subcutaneously in 12 rats

TABLE 2. RESULTS OF COMPLETE STARVATION STUDY

Group	Subcutaneous testosterone propionate implantation	Intrasplenic testosterone propionate implantation	Number of animals	Body weight grams (at start of starvation)	Final body weight, grams	Change body weight, grams	Total prostate weight, mg.	Ventral prostate weight, mg.	Posterior prostate weight, mg.	Milligrams testosterone propionate absorbed per rat per day
WISTAR STRAIN										
Normal diet										
Intact										
A			5	254	257	3	608	377	231	—
Castrate										
B			5	246	261	15	36	18	18	—
C		Yes	4	235	265	30	42	20	22	0.15
D	Yes		6	245	275	30	487	275	212	0.14
Starvation										
Castrate										
E			5	241	157	−84	29	15	14	—
F		Yes	3	235	148	−87	38	16	21	0.14
G	Yes		6	239	158	−81	460	269	191	0.13
SPRAGUE-DAWLEY										
F′		Yes	5	260	172	−88	37	18	19	0.20
G′	Yes		3	275	210	−65	585	382	203	0.15

(Groups D and G) and intrasplenically in 12 (Groups C and F). For the next 4 days, all animals were maintained on 20 gm. stock diet daily. At this time, all food was removed from half the animals with subcutaneous (Group G) and intrasplenic pellets (Group F), as well as those with neither (Group E), and the animals were maintained on tap water only. On the 8th day of starvation, the condition of the animals necessitated cessation of the experiment. The animals were sacrificed by a blow on the head. Adrenal, pituitary and prostatic weights were obtained on a torsion balance. The pellets were removed, cleaned, dried and weighed on a chain balance. Of the original 39 castrates, 31 survived. Of the surviving animals with intrasplenic pellets, 2 (1 in the starved and 1 in the normally fed group) were discarded because of obvious vascular adhesions. During the entire course of this experiment, body weights were obtained at least once weekly and at autopsy.

Eight 200 gm. male rats of the Sprague-Dawley strain were treated in a similar manner. The only exception was that the interval between the implantation of pellets and the onset of the period of starvation was 3 instead of 4 days. The pellets used for subcutaneous implantation (Group F[1]) in this group ranged from 7.3–7.8 mg., and those used for intrasplenic implantation (Group G[1]) ranged from 10.4–17.7 mg.

Results. This data is presented in Table 2. The pellets of testosterone propionate, implanted subcutaneously in both the fed and starved castrates, caused a marked prostatic growth. In no case did intrasplenic testosterone propionate pellets result in a significant increase in the prostatic weight of the castrate rats.

3. *Hypophysectomy.* Rats of the Sprague-Dawley strain were used to test the ability of hypophysectomized animals to inactivate splenic testosterone propionate. Thirty-five hypophysectomized animals were obtained from the Hormone Assay Laboratories, Inc., Chicago. They were to have weighed 200 gm. at the time of hypophysectomy. Five days after hypophysectomy, the animals were castrated. At this time, control

TABLE 3. RESULTS OF HYPOPHYSECTOMY STUDY

Group	Castrate	Subcutaneous testosterone propionate implantation	Intrasplenic testosterone propionate implantation	Number of animals	Initial body weight, grams	Final body weight, grams	Change body weight, grams	Final body length, inches	Total prostate weight, mg.	Ventral prostate weight, mg.	Posterior prostate weight, mg.	Milligrams testosterone propionate absorbed per rat per day
Non-hypophysectomized												
A				5	221	320	99	8.8	662	482	180	—
B	Yes			6	214	316	102	8.8	37	16	21	—
C	Yes		Yes	10	208	297	+89	8.7	48	23	25	0.16
D	Yes	Yes		6	208	320	112	9.0	780	500	280	0.19
Hypophysectomized												
E				4	173	161	−12	7.7	29	13	16	—
F	Yes			2	163	163	0	7.5	30	16	14	—
G	Yes		Yes	7	168	158	−10	7.6	35	17	18	0.15
H	Yes	Yes		4	165	173	8	8.0	474	269	205	0.19

groups of non-hypophysectomized animals were also castrated. All were offered 20 gm. of Purina Laboratory Chow daily. Twenty days after castration, pellets of crystalline testosterone propionate (9.6–15.6 mg.) were implanted intrasplenically and subcutaneously in castrates of both hypophysectomized and non-hypophysectomized groups. Fourteen days later, the animals were sacrificed and organ and pellet weights obtained as before.

Results. The results of this experiment are shown in Table 3. Eight of the castrate-hypophysectomized rats with intrasplenic pellets (Group G) survived to the termination of the experiment. One of these had to be discarded because of vascularized adhesions between the spleen and the abdominal wall, leaving 7. Of interest is the fact that castrate-hypophysectomized rats with subcutaneous pellets of testosterone propionate (Group H) showed an average prostatic weight of only slightly over one-half that shown by nonhypophysectomized castrates (Group D). This was true despite the fact that the total average absorption was the same in both groups.

Other than this, the results were similar to those seen in the other groups. Judging from the prostatic weights of the castrate-non-hypophysectomized rats with subcutaneous pellets (Group D), the amount of androgen being supplied was greater than normally supplied by the testes. Neverthe-

less, there was no evidence of androgenic activity in either hypophysectomized or non-hypophysectomized animals with testosterone propionate pellets in the spleen (Groups C and G).

4. *Glucose-inanition.* In the final group of experiments, the ability of rats on normal stock diet to prevent splenic androgen from influencing prostatic growth was compared to that ability in rats maintained on (1) reduced stock diet or (2) dietary intakes of only glucose and tap water. Seventy 200 gm. male albino rats of the Wistar strain were used. Sixty of these were castrated. Forty-eight of these survived. Eleven days after castration, the rats in Groups G, H, I, and J, in Table 4, were begun on a dietary intake of nothing but glucose and tap water. This was continued until the termination of the

TABLE 4. RESULTS OF GLUCOSE-FEEDING STUDY

Group	Subcutaneous testosterone propionate implantation	Intrasplenic testosterone propionate implantation	Number of animals	Initial body weight, grams	Final body weight, grams	Change body weight, grams	Final body length, inches	Total prostate weight, mg.	Ventral prostate weight, mg.	Posterior prostate weight, mg.	Milligrams testosterone propionate absorbed per rat per day
Stock diet											
Intact											
A			4	179	274	95	8.5	600	381	219	—
Castrate											
B			4	208	266	58	8.5	37	21	16	—
C		Yes	5	211	275	64	8.5	49	27	22	0.20
D	Yes		4	225	314	89	8.7	627	413	214	0.11
Inanition											
Castrate											
E		Yes	6	217	137	−80	7.7	36	17	19	0.15
F	Yes		3	203	138	−65	7.7	631	437	194	0.10
Glucose											
Intact											
G			2	208	135	−73	7.7	133	97	36	—
Castrate											
H			3	215	132	−83	7.7	20	11	9	—
I		Yes	12	222	140	−82	7.7	26	12	14	0.15
J	Yes		9	213	134	−79	7.7	537	359	178	0.12

experiment. Rats in Groups E and F were begun on reduced intake of Purina Laboratory Chow in an attempt to keep them in a weight range comparable to rats on a dietary intake of glucose. The amount of stock diet administered daily was varied on this basis and ranged from 5–10 gm. daily. Three weeks after castration and 10 days after the special dietary manipulations were begun, pellets of testosterone propionate were implanted subcutaneously and intrasplenically in groups of castrate rats on (1) 20 gm. stock diet daily (Groups D and C), (2) reduced rations of stock diet (Groups F and E), and (3) glucose and tap water ad libitum (Groups J and I). The pellets implanted intrasplenically ranged from 8.1–14.9 mg.; those implanted subcutaneously weighed 5.4–9.0 mg. at the time of implantation. Nineteen days after the implantation, the animals were sacrificed with ether. At autopsy, body weight, body length, adrenal, pituitary and prostatic weights were obtained. The pellets were handled as before.

In this experiment, groups of normals and castrates on reduced stock diet were omitted because of limited animal space and because previous experience with these groups decreased the necessity for these controls.

Results. Table 4 gives the results for this group. It will be noted that the weight lost by animals on glucose and by those on decreased intake of stock diet was comparable. The rate of absorption from the larger intra-

splenic pellets was greater than that from the smaller pellets implanted subcutaneously. Nevertheless, castrates with pellets in the spleen, regardless of diet or weight loss, failed to show any prostatic growth; whereas the prostatic weight of animals with subcutaneous pellets approached or exceeded the prostatic weight of the normal controls.

DISCUSSION

Throughout this entire series of experiments, certain observations seem consistent. A decrease in prostatic weight appears to be associated in the intact animal with a loss in body weight. This has been explained on the basis of a decreased gonadotropin output by Moore and Samuels (1931). In every instance we have observed that pellets of testosterone propionate implanted subcutaneously in castrates caused an increase in prostatic weight, usually equaling or exceeding the prostatic weight in the normal controls. The castrates on stock diet with subcutaneous pellets and those on dietary deficiencies with subcutaneous pellets usuallly showed prostatic weights in the same range. The only exception is the group of castrate-hypophysectomized males with subcutaneous pellets; in this case, the prostatic weight was about two-thirds of that of the normal control and a little over one-half of that of the non-hypophysectomized castrates with pellets beneath the skin. Since the amount of testosterone propionate absorbed was the same, this may represent a decreased responsiveness to the androgen. This fact is being investigated further.

In contrast to the prostatic growth occasioned by subcutaneous testosterone propionate, the prostatic weights of animals with intrasplenic testosterone propionate were always in the castrate range. This was true despite a rate of absorption equal to or greater than that which, when occurring subcutaneously, caused prostates that were usually at least physiological in size. Using prostatic weight as an indicator, there was no evidence of androgenic activity in those animals with intrasplenic testosterone propionate pellets. Neither carbon tetrachloride poisoning, complete starvation hypophysectomy, inanition or feeding a diet of only glucose and tap water had any effect on the ability of the liver to prevent testosterone propionate from reaching the general circulation in active androgenic form. We have not in our own experiments completely ruled out the role of the spleen in this function, but from previous work it seems that the liver plays at least the major, if not the entire, role. The persistence of this androgen inactivating function, in the face of liver toxins and dietary stress, makes us agree with Biskind (1943) that the mechanism by which the liver handles testosterone propionate differs markedly from that by which it handles estrogen. It is suggested from this remarkable persistence that the liver is a "physiological filter" for testosterone propionate and probably for other

androgens. The intact normal organ probably has the ability to rid the body of many times the amount of androgen it is normally called upon to handle.

The method by which the liver rids the body of circulating androgen is not clear. Samuels (1949) using *in vitro* techniques gathered evidence suggesting that a multiplicity of liver enzyme systems was concerned with the destruction of testosterone. He demonstrated in rats that complete inanition for 48 hours, as well as niacin tryptophane deficient diets, markedly reduced the ability of liver samples to metabolize testosterone. Despite this marked reduction, the amount destroyed was not inappreciable. In our experiments, we found that neither starving for 8 days nor restricting the dietary intake to only glucose and tap water for 29 days had any effect on the ability of the liver of the intact rat to prevent splenic testosterone propionate from entering the general circulation as an active androgen. Moderately severe parenchymal damage with carbon tetrachloride did not affect this ability. The remarkable resistance of this process to various severe stresses suggests a multiplicity of pathways. In addition to the various metabolic pathways, the biliary excretory pathway demonstrated by Paschis, *et al.* in dogs (1944) may play an important part in the intact rat under some conditions. The important fact is that the intact liver of the rat is apparently able to prevent appreciable amounts of splenic testosterone propionate from entering the general circulation as an active androgen even when the whole organism is subjected to toxic and dietary stresses.

SUMMARY

Pellets of testosterone propionate implanted in the spleen of castrate male albino rats failed to have an effect on prostatic weight when the rats were subjected to the following procedures thought to affect liver metabolism: (1) carbon tetrachloride poisoning, (2) starvation for 8 days, (3) hypophysectomy, (4) moderate inanition, and (5) a restriction of dietary intake to glucose and tap water only for 29 days. Under the same conditions pellets of testosterone propionate implanted subcutaneously always resulted in a marked increase in prostatic weight. These results differ from those reported for estrogens. It seems that even under severe stress the liver is a "physiological filter" for testosterone propionate and probably for other androgens.

Observations suggesting that the prostates of hypophysectomized rats were less responsive to testosterone propionate than those of non-hypophysectomized rats were made.

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THE EFFECT OF GROWTH HORMONE ON GLYCOSURIA IN THE DIABETIC RAT[1]

JANE A. RUSSELL[2]

Department of Physiological Chemistry, Yale University School of Medicine, New Haven, Conn.

IN THE normal cat or dog, the diabetogenic effects of repeated injections of whole anterior pituitary extracts may be reproduced by the administration of highly purified growth hormone (Cotes, Reid and Young, 1949; Houssay and Anderson, 1949; Campbell *et al.*, 1950a, 1950b). Adrenocorticotrophic hormone (ACTH) appeared relatively ineffective as a diabetogenic agent in dogs (Campbell *et al.*, 1950; Houssay and Anderson, 1949). In the rat, on the other hand, ACTH (or cortical extract) readily augments existing glycosuria in diabetic animals and may induce glycosuria in the force-fed intact rat, but convincing evidence of diabetogenic activity attributable to the action of growth hormone has been difficult to obtain in this species. With partially purified growth hormone preparations, Marx *et al.* (1943) observed increases in the glycosuria of sucrose-fed partially depancreatized rats, and more recently Gaarenstrom *et al.* (1949) reported some increase in the glycosuria of fed alloxan-treated rats which were very mildly diabetic but not in those already excreting any considerable amounts of glucose. The doses of the partially purified materials used by these workers were of the order of 5 to 10 mg. per day per animal. Bennett and coworkers (1947, 1948a, 1948b) gave highly purified growth hormone in doses of 3 mg. per day to rats with alloxan diabetes of moderate severity. The average amounts of glucose excreted during the period of treatment were increased significantly in some of the animals given stock diets but not in those fed low carbohydrate diets, nor in hypophysectomized diabetic rats. In the same circumstances, the administration of a like amount of ACTH consistently increased the glycosuria to a considerable extent. The present observations report the results of some further attempts to increase the glycosuria of diabetic rats by the administration of highly purified growth hormone.

METHODS AND MATERIALS

The animals used in these experiments were young adult male rats of the Sprague-Dawley strain. Diabetes was induced by the administration of alloxan (170 mg. per kg.

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[2] Present address: Department of Biochemistry, Emory University, Ga.

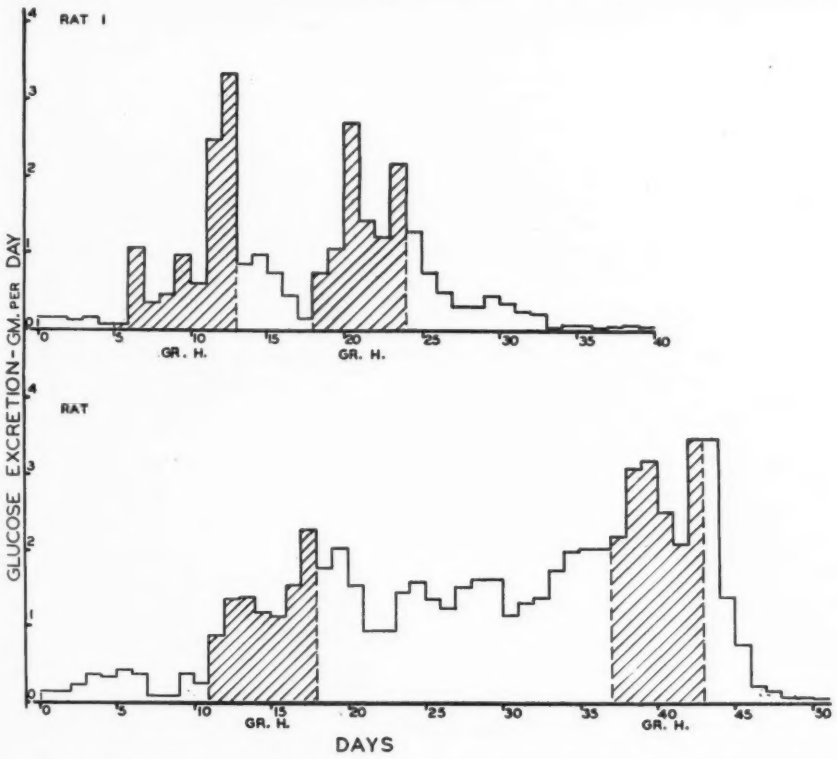

FIG. 1. The effect of growth hormone on the glycosuria of rats. The observations recorded in the chart were begun 31 and 35 days respectively after the administration of alloxan. Rat No. 1 weighed 350 gm. at the beginning of the treatment and was given 20 gm. of food (dry) per day. Rat No. 2 weighed 330 gm. and ate on the average 25 gm. of food per day.

subcutaneously or 40 mg. per kg. intravenously). One to two weeks after the induction of diabetes, the animals were placed in metabolism cages and daily measurements of food intake and glucose excretion were begun. The diet used consisted of Purina Laboratory Chow, ground and mixed to a paste with an equal weight of water. When both appetite and degree of glycosuria had become fairly constant (usually about a month after the injection of alloxan), the animals thereafter were given daily an amount of food equal to the average voluntary food intake in the immediately preceding period. In most instances, the animals ate all of the food given. After a further period of several days, the treatment with growth hormone was begun, the same amount of food being given to each rat before, during, and after treatment. Data for the latter periods of observation only have been included in Figs. 1 and 2.

The growth hormone preparations used were made by the method of Wilhelmi, Fishman, and Russell (1948). They were electrophoretically homogeneous and contained

no detectable amount of ACTH. The hormone was injected intraperitoneally in saline, usually in a volume of 0.2 ml. per 100 gm. body weight. In most instances the total dose was given once daily, but in some animals, two injections were made each day. In the limited number of experiments performed, no differences in response could be attributed to this difference in frequency of injection of the hormone.

The urine of the animals was collected daily under toluene in the presence of a small amount of sulfuric acid. After measurement of the volume of the urine, filtration, and dilution of an aliquot to an appropriate volume, the carbohydrate content of the urine was determined by the anthrone reaction. The conditions used in the analysis were essentially those described by Morris (1948). In a number of urine samples, containing varying amounts of glucose, determinations were made both by the anthrone reaction and by the Benedict method; the results were entirely parallel, but those obtained by the anthrone reaction averaged about 12 per cent higher than those obtained by the Benedict method. By both methods, normal rats under these conditions appeared to excrete 20 to 80 mg. of glucose daily. The only difficulty encountered in the use of the convenient anthrone reaction for the estimation of urine glucose was that in a few of the

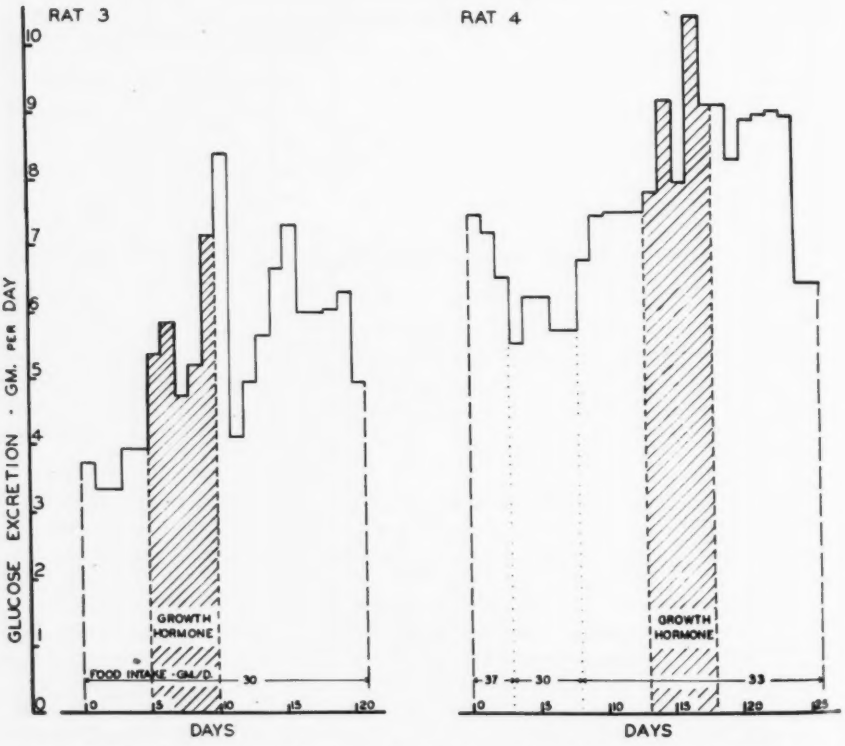

FIG. 2. The effect of growth hormone on the glycosuria of rats with moderately severe diabetes. The observations reported were begun 70 and 62 days after alloxan respectively. Both animals weighed 355 gm. at the beginning of treatment.

more severely diabetic rats the urine contained some substances which gave a brown color as well as the blue color characteristic of carbohydrates. Such urine samples usually contained protein, and the brown color was reduced by prior precipitation of protein with trichloracetic acid. If the samples were then diluted so as to contain around 10 μg. of carbohydrate per ml. and the colors read at 620 mμ, interference by the extraneous color was minimal.

Blood glucose estimations were made at intervals before, during, and after treatment of the animals observed. Usually the samples were taken while the animals were in the unfasted state, but in some instances fasting blood sugar values also were obtained. The blood was taken from the cut tail without anesthesia, and 0.1 ml. samples were employed for analysis by the Nelson-Somogyi method.

RESULTS

In Fig. 1 and Fig. 2 are presented the results of the administration of growth hormone in doses of 4 to 5 mg. per 100 gm. per day to 4 alloxan diabetic rats. In animals No. 1 and 2 (Fig. 1), the degree of glycosuria immediately before treatment was minimal. Both animals had excreted some glucose (up to 1 or 2 gm. per day) during the first weeks after alloxan, but for two weeks prior to the administration of growth hormone had excreted no more than 0.5 gm. per day. During the pre-treatment period, the blood glucose levels in the fed and fasted states were within the normal range, but the glucose tolerance curves were elevated. As shown in the chart, in both instances treatment with growth hormone was accompanied by an immediate increase in the amount of glucose excreted. After the cessation of treatment in the first animal, glucose excretion fell to a low level and was then resinstated by a further course of treatment. In the second animal, the amount of glucose excreted remained elevated after treatment was stopped. Here, a second course of injections further increased the glycosuria, but when treatment was stopped again, glucose excretion fell rapidly to insignificant amounts. Observations (not recorded in the chart) were continued on this animal (No. 2) for an additional 36 days. During this time the glycosuria continued to be slight except that 1 gm. of glucose was excreted on the day after a single injection of growth hormone (16 days after the last previous injection).

The effects of growth hormone on two rats with more severe diabetes are presented in Fig. 2. The observations recorded were begun 70 and 62 days respectively after alloxan, and treatment with growth hormone started at 75 days in both animals. In animal No. 3, the amount of glucose excreted in the preliminary periods had been at no time greater than 4 gm. per day, and during most of the time (with a food intake somewhat smaller than that taken during the recorded period) from 1.5 to 3 gm. per day was the usual amount. During the 5 day period of treatment, the glycosuria increased considerably, and it remained generally high for some time thereafter.

It would have been of interest to observe in such an animal the results of more prolonged treatment; from the further increase in glucose excretion seen on the first day after treatment was stopped, it seems likely that continued injections might have led to progressively more severe diabetes.

Animal No. 4 presented a similar picture. This animal had varied its voluntary food intake rather widely during the preliminary periods, with corresponding fluctuations in glycosuria, and part of these changes are demonstrated in the chart. Even with maximum food intake, however, the amount of glucose excreted had at no time exceeded 7.5 gm. per day. With growth hormone, the rate was increased to 9 to 10 gm. per day during the latter part of the period of treatment, and it remained near this level during a further 6 day period of observation. The fasting blood glucose determined 2 weeks before the injections were begun was 122 mg. per cent. A week after the cessation of treatment, the blood glucose after a 24-hour fast was 213 mg. per cent.

In addition to the experiments recorded in the figures, five other similar series of observations were made. In the first of these, on a rat which was excreting a small amount of glucose before treatment, a series of preliminary observations were made in which different doses of growth hormone were employed. Initially, 1 or 2 mg. of hormone per 100 gm. was given daily without clear-cut effect. Later the dose was increased to 5 mg. per 100 gm., and an increase in the amount of glucose excreted, resembling that shown for animals no. 1 and 2, was seen. After this treatment the animal continued for some time to excrete somewhat more glucose than it had before. Several weeks later, a further course of treatment (4 mg. per 100 gm. per day) was accompanied at first by increased glycosuria, but after a few days, the glucose excretion declined, and after treatment was stopped the animal remained completely aglycosuric. Two other rats which also were excreting very little glucose were given growth hormone (3 to 5 mg. per 100 gm. per day), but without any evident effect. However, both of these rats ate relatively little food, about 9 gm. per day for one and 12 gm. for the other, instead of the 20 gm. or more usually taken; so the lack of effect may have been explicable on this basis. A fourth rat was severely diabetic, excreting 9 to 12 gm. of glucose daily. In this animal, growth hormone given in a dose of 2 mg. per 100 gm. per day was without significant effect on the glycosuria. It might have been expected that a more severely diabetic animal would be sensitive to smaller doses of growth hormone, but evidently this was not the case in this animal.

The last of these five animals was one which had been severely diabetic and then had been hypophysectomized. No significant amounts of sugar were excreted after the first few post-operative days, nor did a course of injections of growth hormone (5 mg. per 100 gm. per day) begun 48 days after

hypophysectomy, effect any degree of glycosuria. However, the blood glucose level in the unfasted state was increased from 137 mg. per cent before treatment to 161 mg. per cent on the third day and 209 mg. per cent at the end of 5 days of injections. (The samples in each case were taken 24 hours after the last injection.) Four days after the cessation of treatment, the blood glucose had returned to 129 mg. per cent. A second course of treatment some weeks later had substantially similar results. The growth hormone seemed thus to have induced some elevation in the prevailing blood glucose levels, but this increase was insufficient to produce glycosuria.

Evidence that even a single injection of growth hormone may increase the severity of the diabetes in susceptible animals was obtained inciden-

TABLE 1. THE EFFECT OF A SINGLE INJECTION OF GROWTH HORMONE ON THE FASTING BLOOD GLUCOSE IN ALLOXAN-DIABETIC RATS

Rat No.	Fasting blood glucose (mg. per cent) determined at weekly intervals		
	Before treatment	After treatment	Av. diff.
1	110, 183, 154, 167*	333, 249, 373, 351	+173 ± 30
2	179, 159, 142, 172*	296, 271, 351, 310	+144 ± 19

* Growth hormone (3 mg. per 100 gm.) given after this observation.

tally in the course of some other experiments. In this work, the fasting blood sugar levels of diabetic rats were obtained at approximately weekly intervals before and after the administration of growth hormone. The data obtained on two of these animals in presented in Table 1. The initial observations on these rats were made 7 weeks after the injection of alloxan, when the blood glucose values seen in the fed state were 428 and 402 mg. per cent, and after fasts of 18 hours, 110 and 179 mg. per cent, respectively. As shown in the table, the fasting blood sugar levels remained in the range of 140 to 180 mg. per cent for the next several weeks. Immediately after the last of the observations recorded, a single injection of growth hormone, 3 mg. per 100 gm., was given to each animal. During the next two hours, the blood glucose rose moderately, as previously described (Milman and Russell, 1950), and the action of a test dose of insulin given two hours after the growth hormone was diminished. Food was given again to the animals a few hours later. A week later, the blood glucose levels of these animals were greatly elevated, and they were seen to remain in the range of 250 to 350 mg. per cent for a period of 5 weeks after the administration of growth hormone. In addition to the two animals just described, 4 others also were given single injections of growth hormone. None of these animals showed the striking rise in fasting blood glucose exhibited by the others, although some evidenced temporary increases of minor degree. It may be significant

that the fasting blood sugar levels of these animals all were lower than in the previous two rats, ranging from 82 to 117 mg. per cent. Of a large number of rats with diabetes of varying degrees of severity which were not given growth hormone, none showed any tendency to become more diabetic but instead often appeared to improve (an observation confirmatory of that made by Wrenshall *et al.* (1949)). The elevation in fasting blood glucose in the animals described above would then appear to have been significant.

DISCUSSION

In 5 diabetic rats which were ingesting adequate and constant amounts of a stock diet, the administration of growth hormone in doses of 4 to 5 mg. per 100 gm. per day was accompanied by increased excretion of glucose. The inconstant effects seen by Bennett and Li (1947) in similar experiments probably may be attributed to the smaller dosage of hormone employed by these investigators, 3 mg. per day per animal or about 1.5 mg. per 100 gm. daily. The use of higher dosages here had been suggested by the results of earlier experiments (Milman and Russell, 1950) in which it was found in fasting diabetic rats that immediate hyperglycemia was produced readily by 3 to 5 mg. per 100 gm. of hormone but not by 1 or 2 mg. Thus, highly purified growth hormone was found to be an effective hyperglycemic agent both in acute and in chronic experiments when it was given in doses of the same order of magnitude. It seems probable that the same mechanisms were responsible for this action in the two types of experimental conditions and that the immediate hyperglycemia was a phase of the production of the more familiar "pituitary diabetes."

The course of the diabetogenic effect of growth hormone in the present experiments resembles strongly that previously described as following the administration of whole saline extracts of beef anterior pituitary glands to partially depancreatized rats (Long *et al.*, 1940). In the latter preparations, as in the present experiments, the glycosuria continued to increase over several days, and on cessation of treatment, in some instances subsided or in others remained at high levels indefinitely. To judge from the amounts of growth hormone now known to be obtainable from beef pituitaries,[3] and also from the glycostatic activity of whole extracts, it would appear that the order of dosage with growth hormone in the earlier experiments must have been similar to the amount found to be effective in the present series. Since whole saline extracts of beef pituitaries usually contain little

[3] A saline or alkaline extract of fresh ground anterior pituitary lobes from cattle may be expected to contain on the order of 10 mg. of growth hormone per gm. of tissue extracted.

adrenotrophic hormone (unpublished observations), it may be supposed that at least a considerable part of the diabetogenic activity of the crude pituitary extracts was due to the presence of growth hormone in the preparations.

It is to be noted that the amount of growth hormone required to augment the glycosuria of the diabetic rat (3 to 5 mg. per 100 gm. per day) appears to be several times as great as that which is effective in inducing diabetes in the intact cat or dog (from 1 to 3 mg. per kg., approximately). The "diabetogenic" dose in the rat also is large compared to the amount of growth hormone required to increase the rate of growth of adult normal rats or to induce nitrogen retention in acute experiments (about 0.1 mg. per 100 gm.). However, other effects of growth hormone on carbohydrate metabolism may be obtained with smaller amounts of hormone—a dosage of 0.1 to 0.2 mg. per 100 gm. permitting the maintenance of the glycogen content of muscle in the fasting hypophysectomized rat (Russell and Wilhelmi, 1950) and promoting increased deposition of muscle glycogen in the glucose-fed intact animal (Illingworth and Russell, 1950). Thus the rat appears to be able to maintain normal blood glucose levels at a time when growth hormone clearly is affecting other phases of carbohydrate metabolism, and to exhibit hyperglycemia only after the administration of relatively large amounts of the hormone. The difference in sensitivity to the hyperglycemic action of growth hormone of the rat, on the one hand, from the cat or dog, on the other, is not known. It has been suggested that the pancreatic "reserve" of the rat is much greater than that of the cat or dog, so that the rat is able to increase its secretion of insulin in conditions in which other animals are unable to do so. Such an increase in insulin secretion after growth hormone would have to represent a specific response to growth hormone rather than a response to hyperglycemic agents in general, for no marked differences between the rat and the cat or dog have been evident with respect to sensitivity to ACTH or to epinephrine. It now appears that relative insensitivity to the diabetogenic action of growth hormone persists in the rat lacking the greater part of its islet function. Additional differences between the species than the response of the pancreas may determine the effectiveness of growth hormone as a diabetogenic agent, and these remain interesting possibilities for investigation.

SUMMARY

In alloxan-diabetic rats fed constant and adequate amounts of food, the administration of purified growth hormone in amounts of 3 to 5 mg. per 100 gm. per day for several days was accompanied by increased excretion of glucose. After cessation of treatment, the glycosuria in some cases re-

ceded, or in others remained high for some time. In some susceptible animals, a single injection of growth hormone was followed by persistent elevation of the blood glucose level.

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THE RAPIDITY OF THE ADRENOCORTICOTROPIC RESPONSE OF THE PITUITARY TO THE INTRAVENOUS ADMINISTRATION OF HISTAMINE[1,2,3]

WILLIAM D. GRAY[4] AND PAUL L. MUNSON[5]

Department of Pharmacology, Yale School of Medicine, New Haven, Connecticut

INTRODUCTION

IT HAS been demonstrated repeatedly (Sayers, 1950) that under certain conditions, denoted "stressful," the pituitary store of adrenocorticotropic hormone (ACTH) becomes depleted, the ascorbic acid and cholesterol concentrations in the adrenal are decreased, increased amounts of adrenal cortical steroids are liberated, and the adrenal cortex increases in weight; thus indicating that increased amounts of ACTH have been delivered into the blood stream. Nevertheless, attempts even to detect, much less quantitate, ACTH in the blood under any circumstance by administration of serum to hypophysectomized rats have, with two possible exceptions (Cooke, Graetzer, and Reiss, 1948; Taylor, Albert, and Sprague, 1949), been unsuccessful. It would be fruitless to attempt to assay the ACTH concentration in blood under basal conditions until its presence had first been demonstrated unequivocally under conditions of stress. Although the larger amounts presumably released into the circulation at this time should simplify the analytical problem, other difficulties remain.

When ACTH (hog) was administered intravenously in large doses to human subjects it was found that it disappeared almost entirely from the blood within a few minutes (Sayers *et al.*, 1949). If ACTH of endogenous origin is subject to a similar type of dissipation, its increased concentration in the blood following stress may be very transitory. Thus, in order to

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[1] Part of a dissertation presented by William D. Gray for the degree of Doctor of Philosophy in Yale University.

[2] Read in part at the 1950 Meeting of the American Society of Pharmacology and Experimental Therapeutics (*Federation Proc.* **9**: 278. 1950).

[3] These studies were aided by a grant from special funds allocated to the Metabolism and Endocrinology Study Section (Dr. Joseph H. Barach, Chairman), U. S. Public Health Service, for the study of anterior pituitary hormones, by a contract between the Office of Naval Research, Department of the Navy and Yale University (NR 113-018), and by a gift from The Wilson Laboratories.

[4] Present address: Pharmacology Research Department, Lederle Laboratories.

[5] Present address: Harvard School of Dental Medicine.

estimate the ACTH concentration of the blood at its peak following a specified stressful stimulus, it becomes important to know how soon after application of an effective stimulus the pituitary begins to respond by accelerating its release of ACTH. Otherwise, the assays for ACTH might fail either by withdrawal of blood for assay before the pituitary response has begun, or so late that the ACTH release has not only begun but has also ended and the transient excess of ACTH in the blood already disappeared.

The results of this study of the rapidity of ACTH release by the pituitary in response to stress have not only clarified our approach toward the problem of measurement of the ACTH concentration in blood but also have raised some interesting questions concerning the mechanism by which stressful stimuli act upon the pituitary.

Sayers and Sayers (1947) found that the reaction of the pituitary of the rat to stimuli of moderate intensity, such as an appropriate dose of histamine, can be prevented by pre-treatment of the animal with cortical hormones or adrenal cortical extract (A.C.E.). That the locus of the inhibitory action is not in the adrenal is evident from the fact that administration of A.C.E. has no influence on the adrenal ascorbic acid depletion caused by an injection of ACTH. These findings provided the basis for our method of investigation of the time relation between application of stimulus and response. Histamine was selected as the stimulus because its effect had already been shown by others to be susceptible to inhibition by cortical hormones, because its effect is transitory and unlikely to outlast the influence of the inhibitor, and because it can be administered intravenously. Intravenous administration is important because the factor of delayed absorption by other routes is absent and the time of effective application can be accurately determined. The criterion for a significant increase in rate of release of ACTH by the pituitary was a decrease in adrenal ascorbic acid concentration one hour after stimulation.

First, the dose of histamine necessary to cause a moderate but not maximal decrease in adrenal ascorbic acid concentration was determined. Secondly, the amount of A.C.E. necessary to prevent adrenal ascorbic acid depletion when administered intravenously shortly before the histamine was determined. Finally, the same amount of extract was administered at varying time intervals after the histamine. If no decrease in adrenal ascorbic acid concentration occurred, the "reaction time" of the pituitary must have been *slower* than the corresponding time interval between injection of the histamine and injection of the inhibitory A.C.E. Conversely, if A.C.E. did not prevent or influence the degree of adrenal ascorbic acid depletion expected from histamine, it could be concluded that

the "reaction time" was *faster* than the time interval between the two injections.

METHODS

Albino rats (mainly males) of an inbred strain descended from Sprague-Dawley ancestors and obtained from the Holtzman-Rolfsmeyer Company[6] were used in all experiments. They were held in the laboratory for 5 to 10 days in order to allow complete recovery from the stresses incident to travel; and because this period appeared ample for adaptation to their new environment. During this holding period they were also handled frequently in order that they might become accustomed to the experimenter and not become unduly excited later during the administration of an anesthetic. The rats varied from 35 to 45 days of age and from 80 to 120 gm. in weight, but in each individual experiment they were of the same age and sex. They also came from the same shipment, and their body weights varied within a narrow range. Purina Laboratory Chow and water were provided *ad libitum* at all times.

The experiments were carried out according to the randomized "block" or group design (Fisher, 1949); thus any possible effect of the order in which the rats were subjected to their assigned treatments could be eliminated from the interpretation of the results.

All experiments were performed under sodium pentobarbital[7] anesthesia. A freshly prepared, 1 per cent solution in 0.9 per cent saline was administered intraperitoneally at 38.5° C. at a dose level of 5 mg./100 gm. Rats which protested administration of the anesthetic were removed from the experiment and not used again for several days. After anesthesia appeared to be complete, in about 10 minutes, the rat's tail was submerged in a constant temperature bath at 38.5° C. for 2 to 5 minutes in order to produce dilatation of the tail veins. Injection of the test solution was then made into a tail vein with a one-half inch, 26-gauge needle. Any evidence of insufficient depth of anesthesia, such as struggling, restlessness, or incomplete flaccidity of the tail during injection was sufficient cause for elimination of the rat from the experiment. In some experiments which required the injection of two different solutions in rapid succession, two syringes were used, the needle being left in the vein while the first syringe was removed and the second one substituted. In other experiments of this type, a two-way adapter was used with both syringes in place, and a correction of 0.1 ml. allowed for the dead space of the adapter. The histamine and other drugs used as stimuli were administered in a volume of 0.2 ml./100 gm. body weight and were injected as rapidly as possible. One-half the dose of A.C.E. was injected in 2 to 3 seconds, and the remainder was injected more slowly so that the total injection time was 10 seconds.

One hour after the intravenous injection, both adrenal glands were removed under sodium pentobarbital anesthesia, carefully but promptly freed of extraneous tissue and weighed to the nearest 0.1 mg. on a micro torsion balance (Roller-Smith). They were then submerged in 0.5 ml. of 2.5 per cent metaphosphoric acid solution contained in a 15×150 mm. heavy wall Pyrex ignition tube (serving as a Potter-Elvehjem (1936) homogenizer) and stored at −10° C. until ready for analysis for ascorbic acid content by the method of Mindlin and Butler (1938). (The procedure will be described in more detail in a subsequent paper (Munson, Barry, and Koch).)

[6] Madison, Wisconsin.

[7] Abbott Laboratories "Nembutal Sodium."

EXPERIMENTAL RESULTS

A dosage level of histamine was sought which would cause a marked difference in adrenal ascorbic acid concentration between the treated animals and controls. At the same time it was desired to avoid maximal stimulation of the pituitary, so that large amounts of A.C.E. would not be required to effect essentially complete inhibition. An extensive series of trials showed that a dose of 0.3 to 0.4 mg. of histamine dihydrochloride per 100 gm. body weight was adequate to produce a decrease from the control mean value of 100 to 150 mg./100 gm. in adrenal ascorbic acid concentration. Injection of 0.5 to 1.0 ml. of aqueous A.C.E. (Wilson)[8] was adequate to inhibit the response completely, or almost completely, when administered intravenously 10 seconds before histamine was given by the

TABLE 1. EFFECT OF I.V. HISTAMINE ALONE OR 10 SECONDS FOLLOWING I.V. A.C.E.

Experiment	Treatment	Number of rats	Mean adrenal ascorbic acid	Variance ratio		Segregation of treatment means*
				Exper.	At P	
			mg./100 gm.			
8	A—Histamine,† 0.25 mg.	4	351	For regression: 9.71	At P=0.05: 5.99	
	B—Histamine,† 0.5 mg.	4	292			
	C—Histamine,† 1.0 mg.	4	283			
9 and 10	A—Controls	7	463	For treatments: 25.56‡	At P=0.001: 13.81	At P=0.05: (B) (D) (A). At P=0.02: (B) (D,A).
	B—Histamine, 0.4 mg.	7	303			
	C—A.C.E., 0.5 ml. 10 sec. before histamine, 0.4 mg.	4	379			
	D—A.C.E., 1.0 ml. 10 sec. before histamine, 0.4 mg.	6	417			

All doses indicated were administered per 100 gm. body weight.
* Groups separated by parentheses are significantly different.
† Histamine dihydrochloride.
‡ Omitting group receiving 0.5 ml. A.C.E.

same route. (Twenty seconds elapsed between the beginning of the A.C.E injection and the start of the histamine injection.)

As shown in experiment 8, Table 1, a dosage level of 0.3 to 0.4 mg./100 gm. would be expected to show a marked but not maximal effect, and this dosage range was utilized in subsequent experiments. In the same table, experiments 9 and 10, it may be seen that A.C.E., administered 10 seconds before histamine, exerts a definite inhibitory effect. The results were analyzed by the method of Tukey (1949), which permits an unbiased comparison of three or more group means. This analysis showed that the A.C.E.-treated groups were significantly different both from the group given histamine alone and from the controls, indicating significant but incomplete inhibition. The two dosage levels of A.C.E. were not significantly different in effect. Nevertheless, for subsequent experiments, the

[8] Generously donated by The Wilson Laboratories through the courtesy of Dr. David Klein, General Manager.

1.0 ml. dose was chosen in order to allow a greater opportunity for consistent expression of the inhibitory property.

In the next phase of the investigation, the effects of A.C.E. administration, both before and after histamine stimulation, were observed. In experiment 11, it was found that even after as short a time interval as 10 seconds, the A.C.E. treatment was too late to exert a detectable inhibitory effect. This finding indicated that the histamine stimulus had already provoked the discharge of a sufficient excess of ACTH from the pituitary to cause adrenal ascorbic acid depletion. When the time interval was shortened to 5 seconds, as in experiment 12, a significant, but not complete, inhibition occurred. (The three group means differ significantly at the $P = 0.02$ level of significance.)

TABLE 2. EFFECT OF I.V. ADMINISTRATION OF 1.0 ML. A.C.E. AT VARYING INTERVALS BEFORE AND AFTER I.V. ADMINISTRATION OF HISTAMINE

Experiment	Treatment	Number of rats	Mean adrenal ascorbic acid	Variance ratio		Segregation of treatment means*
				Treatments	At P	
			mg./100 gm.			
11	A—Controls	4	406	9.65	At P=0.05: 5.79	At P=0.02: (B, C,) (A).
	B—Histamine (H),† 0.4 mg.	4	299			
	C—A.C.E. 10 sec. after H, 0.4 mg.	3	303‡			
12	A—Controls	6	450	47.55	At P=0.001: 14.91	At P=0.02: (B) (C) (A).
	B—Histamine, 0.4 mg.	6	266			
	C—A.C.E. 5 sec. after H, 0.4 mg.	6	322			
13	A—Controls	9	436	24.23	At P=0.001: 7.55	At P=0.02: (B) (D) (C,A). At P=0.01: (B,D) (C,A).
	B—Histamine, 0.3–0.4 mg.	9	287			
	C—A.C.E. 2 sec. before H, 0.3–0.4 mg.	9	421			
	D—A.C.E. 3 sec. after H, 0.3–0.4 mg.	9	341			

All doses indicated were administered per 100 gm. body weight.
* Groups separated by parentheses are significantly different.
† Histamine dihydrochloride.
‡ One missing value supplied by missing plots technique (Emmens, 1948).

There remained the possibility that under these experimental conditions a 1.0 ml. dose of A.C.E. was inadequate for inhibition even if it were given in advance of the histamine. To test this possibility experiment 13 was carried out. (The two different dosage levels of histamine used in this experiment were applied in parallel to the three histamine-treated groups, in conformity with the randomized group design.) It was clearly shown that the same dose of A.C.E. which was adequate to inhibit the histamine response when it was administered 2 seconds before histamine gave only partial protection when administered 3 seconds after histamine. The results of these three experiments are summarized in Figure 1.

It was concluded from the foregoing series of experiments that the anterior pituitary of the rat is capable of releasing ACTH in response to histamine stimulation with extreme rapidity. The interval between stimulus and response appears to be of the order of 5 to 10 seconds,—a time re-

lationship which suggests that histamine may act directly upon the pituitary. If, on the contrary, histamine acts to increase the utilization of adrenal cortical hormones by tissues, thus reducing the concentration of these hormones in the blood, and by so doing removes their inhibitory effect on the release of ACTH by the pituitary, as was postulated by Sayers and Sayers (1945, 1948), then the tissues must indeed be capable of withdrawing cortical hormones from the blood with great rapacity and speed.

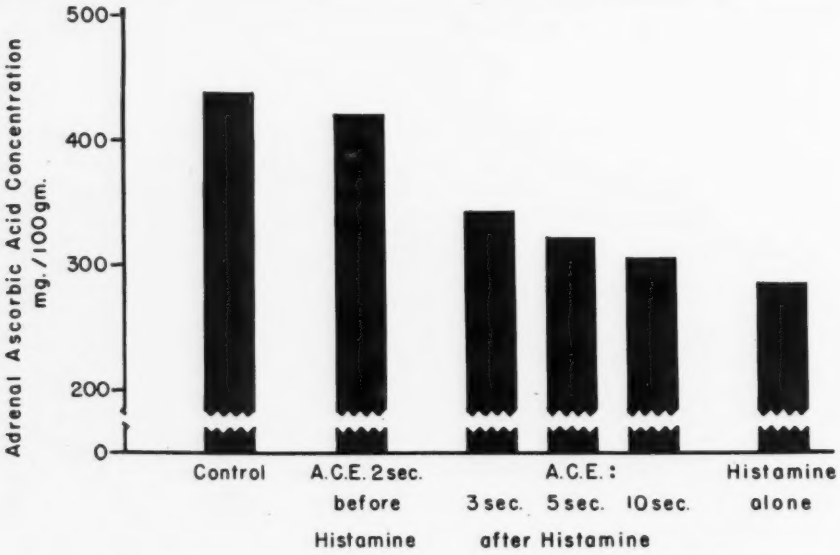

FIG. 1. Effects of A.C.E. on the adrenocorticotropic response of the pituitary to histamine as it is influenced by the order of administration and the time interval between injections of the two agents.

The possibility that the effect of histamine is mediated through the adrenal medulla (Long, 1946, 1947), and that the liberated epinephrine directly activates the anterior pituitary, was investigated in several ways. First, an autonomic ganglion blocking agent, tetraethylammonium chloride[9] (TEAC) (Acheson and Moe, 1946) was administered prior to histamine. Secondly, nicotine, which is known to stimulate release of epinephrine from the medulla, was administered. Thirdly, l-epinephrine itself was injected intravenously.

The results, presented in Table 3, do not lend support to the idea that epinephrine is the primary stimulus for pituitary activation. Experiments 14 and 15 show that TEAC has no inhibitory effect on the histamine re-

[9] Parke, Davis & Company "Etamon."

sponse. It may also be noted that, in confirmation of Tepperman and Bogardus (1948), TEAC itself at this dose level was found to have no effect. In experiment 16, it was found that nicotine was ineffective at dosage levels of 0.0125 and 0.025 mg./100 gm. The effect of 0.05 mg. was significant, but because in our hands doses of 0.06 mg. have been consistently lethal, it is assumed that the effective stimulus, unknown in character, is common to all drugs in the lethal range. All rats treated with nicotine

TABLE 3. ADRENAL ASCORBIC ACID CONCENTRATION FOLLOWING I.V. ADMINISTRATION OF HISTAMINE, TETRAETHYLAMMONIUM CHLORIDE, NICOTINE, AND L-EPINEPHRINE

Experiment	Treatment	Number of rats	Mean adrenal ascorbic acid	Variance ratio		Segregation of treatment means*
				Treatments	At P	
			mg./100 gm.			
14	A—Controls	3	427	33.47	At P=0.001: 23.70	At P=0.05; (C,D) (A,B).
	B—TEAC,† 1.0 mg.	3	441			
	C—Histamine (H),‡ 0.4 mg.	3	235			
	D—TEAC, 1.0 mg., 5 min. before H, 0.4 mg.	3	284			
15	A—Controls	3	425	32.20	At P=0.0001: 23.70	At P=0.05: (D,C) (A,B).
	B—TEAC, 1.0 mg.	3	431			
	C—Histamine, 0.25 mg.	3	298			
	D—TEAC, 1.0 mg., 5 min. before H, 0.25 mg.	3	288			
		3	288			
16	A—Controls	4	399	5.28	At P=0.05: 3.86	At P=0.02: (D) (A,B,C).
	B—Nicotine§, 0.0125 mg.	4	405			
	C—Nicotine, 0.025 mg.	4	414			
	D—Nicotine, 0.05 mg.	4	303			
17	A—Controls	5	477	4.30	At P=0.05: 3.49	At P=0.05: (E) (D,A,C).
	B—l-Epinephrine‖, 0.25 μg.	2	496¶			
	C—l-Epinephrine, 0.5 μg.	5	488			
	D—l-Epinephrine, 1.0 μg.	5	465			
	E—l-Epinephrine, 2.0 μg.	5	387			
18	A—Controls	3	449	0.99	At P=0.05: 8.94	
	B—l-Epinephrine, 0.025 μg.	3	430			
	C—l-Epinephrine, 0.05 μg.	3	430			
	D—l-Epinephrine, 0.1 μg.	3	427			

All doses indicated were administered per 100 gm. body weight.
* Groups separated by parentheses are significantly different.
† Tetraethylammonium chloride.
‡ Histamine dihydrochloride.
§ Nicotine salicylate, dosage as nicotine base.
‖ Synthetic l-epinephrine bitartrate (Winthrop-Stearns) preserved with glutathione, 20 mg./100 ml., dosage as free base.
¶ Omitted from analysis of variance.

showed evidence of respiratory stimulation, salivation, and some degree of cyanosis. Experiments 17 and 18 showed that a wide range of dosages of synthetic l-epinephrine was ineffective. This finding was surprising in view of the well-known effect of epinephrine in stimulating ACTH release when administered subcutaneously, intramuscularly, or intraperitoneally, either rapidly or by slow infusion (Gershberg *et al.*, 1950). The method used for determining pituitary "reaction times" of the order of 5 to 10 seconds required rapid injection, and under these conditions doses greater than 2 μg. were lethal. Even at the 2 μg. level, approximately one-half the rats died. Among the rats who survived this near-lethal dose of epinephrine there was a significant depletion of adrenal ascorbic acid, just as there had been

following the high dose of nicotine. Since an earlier report (Long and Fry, 1945) was based on the use of natural epinephrine which is now known to contain nor-epinephrine (Goldenberg *et al.*, 1949), a further experiment was conducted in which a commercial epinephrine preparation of animal origin[10] was administered. No significant effect on adrenal ascorbic acid concentration was found at the 1 μg. level; and all rats which received 2 μg. succumbed, indicating that our negative results can not be ascribed to an absence of nor-epinephrine from the solution injected. More recently Gershberg *et al.* (1950) have reported that l-nor-epinephrine, injected subcutaneously, has no significant effect on the adrenal ascorbic acid.

In an effort to resolve the apparent inconsistency of these data with those obtained when epinephrine is injected by other routes providing slower absorption, 15 rats were injected intravenously at a slower rate with l-epinephrine. Of 5 rats given a dose of 2 μg. over a period of 10 seconds, three died promptly; but the two who survived showed adrenal ascorbic acid values of 313 and 307 mg./100 gm., respectively. Of 7 rats injected with 1 μg. of l-epinephrine, three yielded values of 219, 286, and 366; while the remaining 4 varied between 464 and 495, i.e., well within the control range. Three rats injected with 1 μg. over a 20-second period all exhibited control values. It would be difficult to assign the positive effects in this group to the specific action of epinephrine, because respiratory embarrassment was common, and this condition, occasionally noted in otherwise untreated animals under pentobarbital anesthesia, is in itself a sufficient cause of depletion of adrenal ascorbic acid.

DISCUSSION

Our results have established the fact that, within 10 to 15 seconds after intravenous histamine administration, an irreversible reaction can be initiated which will eventuate in an increased concentration of ACTH in the blood. It does not necessarily follow that the reaction manifests itself immediately in the form of an increased titer of ACTH in the blood, although possibly it may do so. Allowance also must be made for a circulation time of about 5 seconds between the site of injection and the pituitary (D'Amour and Blood, 1948). (This interval is of no consequence in our method of determining the reaction time of the pituitary since it affects both stimulus and inhibitor equally.) From these considerations it may be concluded that blood samples withdrawn before the lapse of about 15 seconds after histamine injection can not be expected to reveal an increase in ACTH concentration. In any event, obvious technical difficulties would preclude more rapid withdrawal of blood.

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[10] Sharp & Dohme Inc. "Adrin."

Once the increased rate of ACTH release by the pituitary has begun, whether it occurs as a transient burst or as a steady and slow but prolonged acceleration of output, the limited duration of its persistence in the blood stream in an active form must be considered (Sayers *et al.*, 1949). ACTH disappears from the blood by no means instantaneously, however; because we have found (Gray, 1950) that blood drawn from donor hypophysectomized rats 2 to 4 minutes after injection of as little as 10 μg. of standard hog ACTH ("La-1-A") (Munson, Naylor, and Gray, 1950) retained the potency of its hormone content when tested in recipient hypophysectomized rats (Sayers, Sayers and Woodbury, 1948; Munson, Barry, and Koch, 1948).

It is by no means certain that the stability of ACTH of endogenous origin in the rat is identical in the blood stream with that of injected "La-1-A"; but if so, it would seem likely that once the ACTH titer of the blood has reached its peak, it may be expected to persist essentially undimished for 2 to 5 minutes at least. The lability of ACTH in the blood, therefore, should not be an insuperable obstacle in the way of its detection.

In this report the evidence that the pituitary can respond within 10 seconds after the application of stress has been confined to one type of stimulus, namely, intravenous histamine administration. Other drugs in sublethal doses; epinephrine, nicotine, tetraethylammonium chloride, and 2 ml. ice-cold saline, proved to be unsuitable for studies of reaction time because they were not effective as stress agents when injected intravenously. It would be desirable to extend the investigation to other stimuli, such as a purified posterior pituitary extract,[11] which we have recently found to be effective intravenously at a dose level of 0.05 I.U./100 gm. (Munson *et al.*, 1950).

The discovery (Ingle, 1938; Sayers and Sayers, 1945) that treatment with A.C.E. or cortical steroids can prevent the response of the pituitary to certain types of stress strongly suggests that the concentration of adrenal cortical hormones in the blood is important in regulating the rate of output of ACTH by the pituitary. Nevertheless, it is by no means proved that all agents affect the pituitary only by accelerating reactions which deplete the blood of cortical steroids below a certain threshold. At least in the case of histamine stress the reaction time of the pituitary would seem to require clearance of steroids from the blood with greater rapidity than has been thought possible. Long and his colleagues (Long, 1949) have advanced the attractive hypothesis that the ACTH response of the pituitary is sensitive to more than one type of stimulus. Recent experiments reported from Long's laboratory (Gershberg *et al.*, 1950; McDermott *et al.*,

[11] Parke, Davis & Company "Pitressin."

1950) are consistent with the view that epinephrine of medullary origin is responsible for the more rapid pituitary response (the *autonomic* phase); but that a homeostatic mechanism is also active, although more slowly (*metabolic* phase). Our results do not negate the possibility of some such multiple pathway for stressful stimuli. On the other hand, our failure to observe pituitary stimulation following the rapid intravenous administration of sublethal doses of epinephrine, and the finding by Gershberg *et al.* (1950) that slow infusions of physiological amounts of epinephrine intramuscularly are ineffective unless continued for longer than 15 minutes, might lead us to question the physiological significance of the adrenal medulla in the stress mechanism. Other investigators also have uncovered aspects of the pharmacology of epinephrine which do not harmonize readily with the concept that the hormone plays an essential part in the pituitary response to stress (Tepperman and Bogardus, 1948; Ronzoni and Reichlin, 1950; Ronzoni, 1950). It is hoped that new experiments now in progress will resolve inconsistencies which may be more apparent than real. These include investigations of the possible role of vasoconstriction of the blood supply in preventing a reaction to intravenous epinephrine; of the influence of pentobarbital anesthesia in modifying the effects of epinephrine administered by other routes; and of the effect of concurrent administration of epinephrine and histamine.

SUMMARY

The rapidity of the response of the anterior pituitary of the rat (under pentobarbital anesthesia) to an intravenous injection of histamine has been estimated by subsequent administration, at varying intervals, of a uniform dose of adrenal cortical extract of proven inhibitory potency. The occurrence of a pituitary response was established by the decrease in adrenal ascorbic acid concentration one hour after stimulation. No inhibitory action remained after 10 seconds, and inhibition was only partial after intervals of 3 seconds and 5 seconds, respectively. These findings indicate that the "reaction time" of the pituitary under the experimental conditions was of the order of 10 seconds or less.

Nicotine, tetraethylammonium chloride, and natural and synthetic l-epinephrine, when administered intravenously in sublethal doses to the anesthetized rat, failed to produce an accelerated release of ACTH by the pituitary.

Acknowledgment

We greatly appreciate the advice, constructive criticism, and encouragement given us by numerous members of the faculty of Yale School of Medicine, particularly Dr. C. I. Bliss, Dr. D. D. Bonnycastle, and Dr. W. T. Salter; also, we wish to thank Dr. R. O. Greep and Dr. R. J. Barrnett of Harvard University for reading the manuscript.

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NOTES AND COMMENTS

EFFECT OF ADRENALECTOMY AND HYPOPHYSECTOMY ON ACHROMOTRICHIA IN COPPER DEFICIENT RATS

Elaine P. Ralli and associates (1943, 1950) have shown that either adrenalectomy or hypophysectomy will cause the return of black hair in rats made gray by a pantothenic acid deficient diet. This work has led to considerable speculation and experimentation as to a possible role of pantothenic acid in the metabolism of the adrenal cortex. We have been studying the production of gray hair in rats on a copper deficient diet and wish to report the effects of adrenalectomy and hypophysectomy in this type of achromotrichia.

METHODS

Weanling, Long-Evans all black rats subsisting on our copper deficient diet will develop marked graying of the hair in about 8 weeks. This diet is a 20% casein, sucrose, low fat, purified type of diet adequately supplemented with all vitamins known to be required by the rat. The minerals are supplied by Osborne and Mendel salts (1917). This diet and the type of gray hair it produces have been fully described elsewhere (Hundley 1950). Adrenalectomy and hypophysectomy[1] were accomplished using conventional techniques. Adrenalectomized rats were maintained on saline drinking water. At the end of each experiment ordinary tap water was substituted for the saline to discover those animals which failed to show weight loss when deprived of supplemental salt. Such animals presumably had a physiologically incomplete adrenalectomy. Only male rats were used in these experiments.

RESULTS

The influence of the adrenal glands was tested in a preventive experiment designed to determine whether removal of these glands would affect gray hair formation, Table 1. A group of weanling black rats was maintained on the copper deficient diet for 8 weeks. Seventeen animals were adrenalectomized and maintained on saline from the beginning of the experiment. Gray hair was completely prevented in all but 3 which developed 1+, 2+, and 3+ gray hair respectively when graded on an arbitrary scale where 4+ represents maximum grayness under our conditions. The completeness of the adrenalectomy, as judged by the salt deprivation test, had little relation to the results obtained. Eight animals were judged to be complete and one developed gray hair (3+). Nine were judged to be incomplete and two developed gray hair (2+, 1+). None of these rats had visible adrenal tissue when autopsied. None of 6 additional rats which were adrenalectomized and maintained on saline, but which received added copper in their diet, developed gray hair. Twenty-seven littermate controls were subjected to shaving plus sham operation. Some of these received ordinary tap water and some received saline drinking water. Twenty of these developed gray hair of 1+ to 4+ severity. No significant difference in gray hair development was noted between rats receiving tap or saline drinking water. No consistent correlation was noted in any of the groups between the rate of gain on body weight and development of gray hair.

Sixteen male rats in which gray hair was already well developed were used in another experiment to determine whether removal of the adrenals or of the hypophysis would reverse the achromotrichia once it was established. When these rats were shaved over

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[1] We are indebted to Miss Frances Dobell who did the hypophysectomies for us.

the back, very little skin pigment was seen. Each animal usually showed one or two small patches or streaks of faint blue color, the remaining skin being apparently free of pigment. There was no characteristic distribution of the pigment spots, nor was there any relationship between the intensity and location of the pigment and the grayness of the overlying hair. One rat showed no visible skin pigment. Eleven of the gray rats were adrenalectomized. Each animal developed definite regeneration of black hair as evidenced by generalized decrease in grayness or by the appearance of spots of black hair. Eight of these rats were later judged to have physiologically complete adrenalectomies and 6 completely regenerated a normal appearing black hair coat in 38 to 56 days. The remaining two, and the 3 rats judged to have physiologically incomplete adrenalectomies, showed definite but incomplete regeneration of black hair within this period. Each of five gray rats which were hypophysectomized, completely regenerated a normal appearing black hair coat within 35 days.

TABLE 1. PREVENTION OF ACHROMOTRICHIA BY ADRENALECTOMY IN RATS ON A COPPER DEFICIENT DIET

No. rats	Diet	Treatment	No. with gray hair	Grayness index[1]	Wt. gain gm./wk.
19	Cu deficient	Sham operation+tap drinking water	15	1.4	16.5
8	Cu deficient	Sham operation+0.85% NaCl drinking water	5	1.4	13.4
17	Cu deficient	Adrenalectomy+0.85% NaCl drinking water	3	0.4	18.7
6	0.02% $CuSO_4 \cdot 5\,H_2O$ added	Adrenalectomy+0.85% NaCl drinking water	0	0.0	16.2

[1] The Grayness Index was calculated by adding the 0 to 4+ estimates of gray hair severity for each animal and dividing by the number of rats in the group.

The sequence of events was similar after either adrenalectomy or hypophysectomy. The color of the pigment spots deepened and spread rapidly over the entire back so that about 14 days after operation the back was a uniform deep bluish-black color. Hair regrowth occurred first in the spots where pigment was present at the time of operation. This hair was gray, As the pigment spread hair growth began in new areas. Visible hair growth did not appear in any area until skin pigment was visible. This hair was black. The small spots of gray hair which appeared soon after operation were eventually replaced by black hair so that the end result in most animals was a uniform black hair coat.

Gray rats maintained on the copper deficient diet and subjected to shaving, mock operation or given saline drinking water did not show the reaction described above. Hair regrowth was very slow, pigment spread occurred slowly or not at all, the hair which did regrow was generally more gray than the original hair coat. Most of these control animals still had large bald areas devoid of visible pigment after 2 to 3 months.

SUMMARY

Gray hair developed within eight weeks in young black rats maintained on a copper deficient diet. Adrenalectomy completely prevented development of gray hair in most of such copper deficient animals. Either adrenalectomy or hypophysectomy caused the

partial or complete return of a normal appearing black hair coat in rats which had become gray on the copper deficient diet.

These findings indicate a metabolic relationship between copper and the adrenal cortex. They also provide additional evidence of the role of the adrenal cortex in the regulation of hair growth and melanin formation.

JAMES M. HUNDLEY AND ROBERT B. ING

From the National Institute of Arthritis and Metabolic Diseases
National Institutes of Health, Public Health Service
Federal Security Agency
Bethesda, Maryland

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EFFECT OF CORTISONE ACETATE UPON GROWTH OF A LYMPHOSARCOMA* IN THE RAT

The effect of cortisone in suppressing the growth of normal and abnormal tissues has been reviewed (1). Stoerk (5) has reported a marked retardation of growth of lymphosarcoma implants in pyridoxine-deficient rats injected with 2 mg. daily of cortisone acetate. In the present experiments, cortisone acetate was found to retard the growth of a lymphosarcoma in the force-fed rat.

METHODS

Twenty-one male rats of the Sprague-Dawley strain having an initial weight of approximately 300 gms. were used in these experiments. The animals were force-fed 13 cc. per feeding of a medium carbohydrate diet (2) by stomach tube each morning (8:30 to 9:15 A.M.) and afternoon (4:15 to 5:00 P.M.). Following a control period of 14 days, each rat received a small section of the lymphosarcoma (4) by subcutaneous implantation through a trocar high on the right flank. Asepsis was successfully maintained during the preparation and implantation of the tumor. The implants grew in each of the 21 hosts. Three days after the implantation of the tumors, 10 of the rats were given 5 mg. of cortisone acetate (Merck) daily for 8 days. The steroid was ground into a fine suspension in peanut oil, 5 mg. per cc., and was given in divided subcutaneous injections each morning and late afternoon. The 11 control animals received injections of oil only. The animals were killed, and the tumors were weighed and measured on the 8th day following the beginning of treatment with cortisone acetate.

RESULTS

The tumors grew in all of the animals but at a retarded rate in those animals which were treated with cortisone acetate. The average tumor weight for the control animals was 18.3 ± 2.3 gms., and the average tumor weight for the treated series was 8.7 ± 1.8 gms.

DISCUSSION

The results of these experiments are similar to those reported for the effect of cortisone

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* The tumor was obtained through the courtesy of the late Dr. James B. Murphy, Rockefeller Institute for Medical Research, New York.

acetate upon the growth of the Walker Rat Carcinoma under similar conditions of experiment (3).

The thymus and other lymphoid tissue in rats and other animals undergo rapid regression during the administration of large doses of cortisone (1). Under the conditions used here, this lymphomatous tumor was able to remain in an anabolic phase during treatment, although its rate of growth was retarded.

SUMMARY

The administration of 5 mg. of cortisone acetate daily to rats which were hosts to an implanted lymphosarcoma retarded, but did not completely suppress, the growth of the tumor.

DWIGHT J. INGLE AND JAMES E. NEZAMIS

Research Laboratories,
The Upjohn Co.,
Kalamazoo, Michigan

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ACCUMULATION OF RADIOIODINE IN THE THYROID GLAND OF ELASMOBRANCHS[1]

The incorporation of iodine into the thyroxin of the thyroid glands of man and other higher vertebrates has been well demonstrated, but little is known of these relations in the lower vertebrates. Gorbman and Creaser (1942) demonstrated that the endostyle of larval lampreys (ammocetes) can accumulate this element, but they did not mention the concentration of iodine in the adult. Recent development of emulsion-tissue radioautography (Bélanger, 1950) enables us conveniently to demonstrate that the elasmobranch fishes can also concentrate iodine in their thyroids. Elasmobranchs, in contrast with teleosts, possess a large and easily recognized discrete thyroid gland (Gudernatsch, 1911; Ferguson, 1911), which consists of a mass of vesicular follicles lying behind the posterior border of the mandible and resting on the coracohyoid muscle.

In this study three species were used, a shark, *Mustelus californicus*, and two rays, *Zapteryx exasperata* and *Holorhinus californicus*. All were seined near La Jolla, California, and were kept in a cement aquarium with running sea water several days before receiving 400 microcuries of I^{131} intramuscularly near the dorsal fin.[2] Forty-six hours after injection, the thyroid glands were dissected out, and the radioautographic slides were made by the inverted autograph procedure (Bélanger, 1950), in which the emulsion is combined with the tissue on the slide.

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[1] Contributions from the Scripps Institution of Oceanography, New Series, No. 516. Received for publication February 15, 1951.

[2] The I^{131} used in this investigation was supplied by the Oak Ridge National Laboratory on allocation from the Isotopes Division, U. S. Atomic Energy Commission.

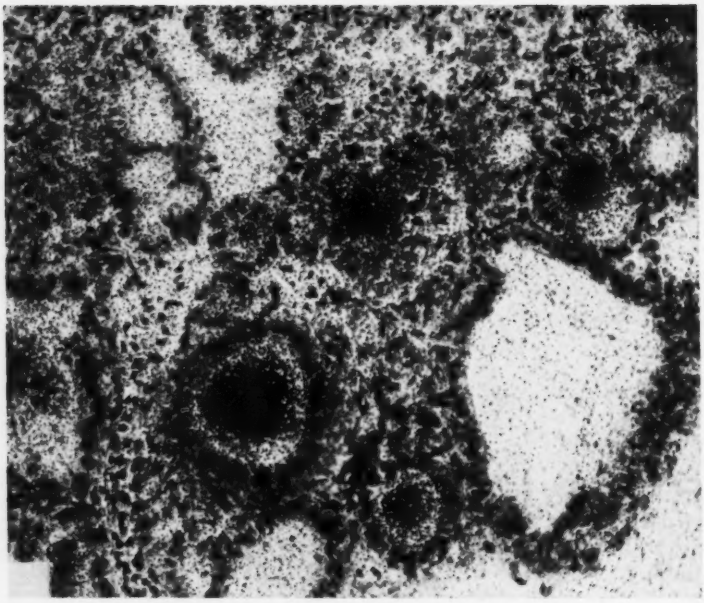

FIG. 1. Portion of thyroid gland of mottled guitarfish (*Zapteryx exasperata*), sacrificed 46 hours after an intramuscular injection of radioiodine. The radio-element is concentrated at the lumen of the follicles. Hematoxylin-eosin. ×300.

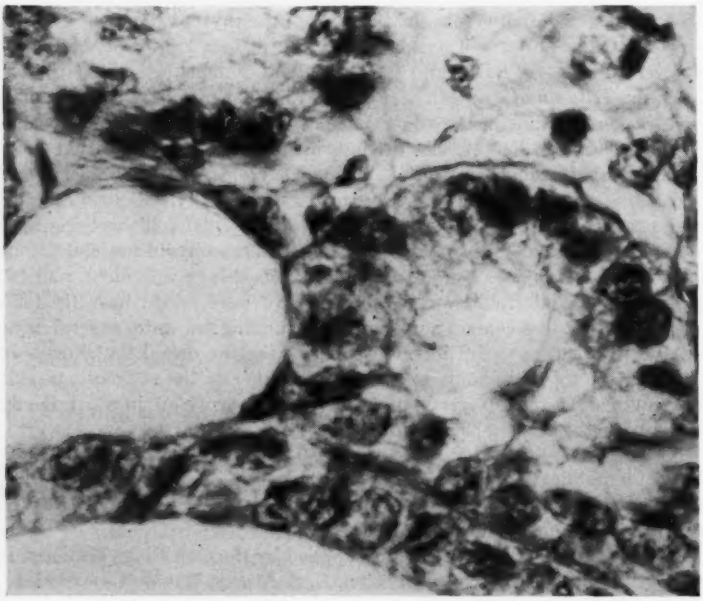

FIG. 2. One small follicle closely adjacent to portion of a large follicle in a thyroid gland of gray smoothhound (*Mustelus californicus*). Note most of the follicular nuclei are faint in Feulgen stain. ×920.

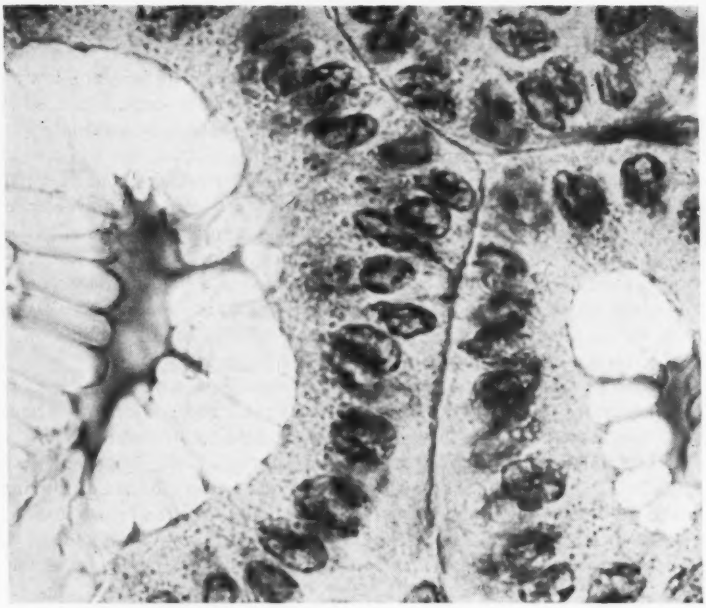

FIG. 3. Three adjacent follicles of an iodine-concentrated thyroid of *Zapteryx exasperata*. Note that the chromatin materials of the follicular nuclei condensed and adhered to the nuclear membranes, and that the nuclear membranes are folded. Feulgen stain. ×910.

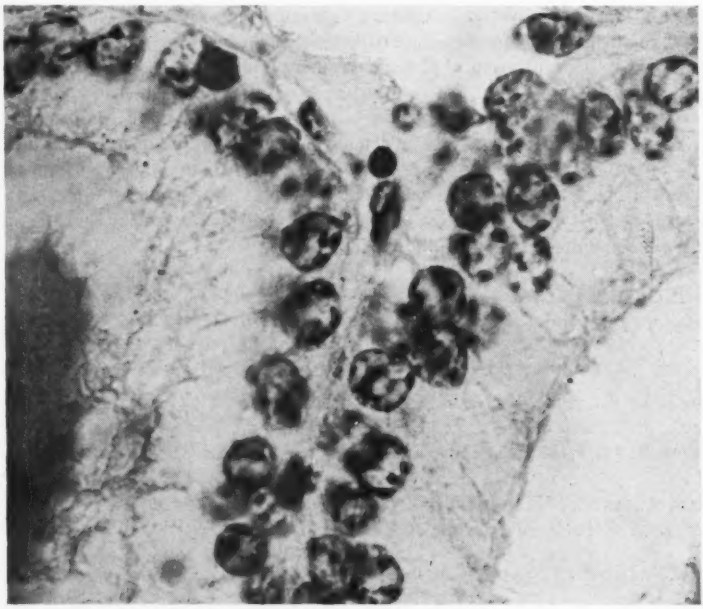

FIG. 4. Portion of two adjacent follicles of a postmortem thyroid taken from a *Zapteryx exasperata* shortly after death. Note that the heterochromatin is highly condensed and intensely stained by the Feulgen dye. ×920.

The vesicles of the elasmobranch thyroids are lined by epithelium of a low columnar type, containing colloid. In the emulsion-tissue slide, little assimilation of iodine is shown in the follicular epithelium. More conspicuous, often, but markedly variable is the concentration of radioiodine in the follicles. In adjacent follicles the uptake of the radioiodine varies greatly (Fig. 1). In general the smaller follicles contain much radioiodine and sharply contrast with adjacent large follicles, which accumulate little iodine. In the same gland the larger follicles may contain more colloid with slight activity, while the smaller ones contain less colloid but more iodine. This may indicate, in the elasmobranchs as in other vertebrates, that the volume of colloid in the thyroid follicles cannot be regarded as a reliable index of the activity of the secretory epithelium. An estimate of the secretory activity of the glandular follicles could be based on the concentration of radio-activity shown in the inverted autograph (Fig. 1).

Destruction of normal thyroid gland by the administration of I^{131} has been reported in higher vertebrates (Goldberg, Chaikoff, Lindsay, and Feller, 1950, with references). The 400-microcurie dose of this isotope in the elasmobranch thyroid similarly induced swelling and vacuolation of the cytoplasm of many epithelial cells. Degenerative changes were also visible in the nuclei. The nuclear membrane became folded and wrinkled, as it did in the rat thyroid as described by Goldberg, *et al.* In the normal thyroid gland, as determined in *Mustelus californicus*, the chromatin materials of the nucleus were less basophilic and were faint to the Feulgen test (Fig. 2), obviously because of the prevalence of ribonucleic acid in the nucleus. However, in the follicular nucleus of the irradiated thyroid of *Zapteryx*, some chromatin was condensed into a little lump of the Feulgen-positive desoxyribonucleic acid adhering to the nuclear membrane (Fig. 3). This phenomenon became more manifest as the heterochromatin lumps were highly condensed and intensely stained by the Feulgen dye, as was seen in a thyroid taken from a *Zapteryx* shortly after death (Fig. 4).

Radiation is known to produce minute nuclear and cytoplasmic changes. The first effect seems to be the transformation of ribonucleic acid into desoxyribonucleic acid, and involves a reversible inhibition of the synthesizing abilities of desoxyribose acid within the cells. With this inhibition the supply of ribose nucleotides to the cell metabolism would depend on the synthesis of cytoplasmic proteins. But the radiation probably would exert the same degenerating effect in cytoplasm. The cytoplasmic ribonucleic acid supply to the nucleoprotein synthesis can no longer be metabolically interchangeable, thereby initiating the cellular degeneration process in the thyroids.

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TEH PING LIN AND EDWARD D. GOLDBERG

Scripps Institution of Oceanography,
University of California

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